STRUCTURE ACTIVITY RELATIONSHIP FOR THE NEUROPROTECTIVE EFFECTS OF ESTROGENS: POTENTIAL INVOLVEMENT OF cAMP RESPONSE ELEMENT BINDING PROTEIN AND NUCLEAR FACTOR KAPPA B

By

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for my grandmother,
Pattie L. Kopcak

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KEY TO ABBREVIATIONS

αE2	17α-estradiol
βE2	17β-estradiol
Aβ	β-Amyloid Peptide
AC	Adenylate Cyclase
AD	Alzheimer's Disease
AMPA	α-amino-3-hydroxy-5-methyl-4-isoazolepropionate
ATF1	Activating Transcription Factor 1
APP	Amyloid Precursor Protein
BDNF	Brain-derived Neurotrophic Factor
BSA	Bovine Serum Albumin
CAMK	Ca^{2+}-Calmodulin Kinase
cAMP	Cyclic Adenosine 3',5'-Monophosphate
CBF	Cerebral Blood Flow
CEE	Conjugated Equine Estrogen
CORT	Corticosterone
CRE	Cyclic AMP Response Element
CREB	Cyclic AMP Response Element Binding Protein
E-3-ol	Estratriene-3-ol
EAA	Excitatory Amino Acids
EGF	Epidermal Growth Factor
ER	Estrogen Receptor
ERE	Estrogen Response Element
ERK	Extracellular-signal Regulated kinase
ERT	Estrogen Replacement Therapy
FBS	Fetal Bovine Serum
γGCS	γ-Glutamylcysteine Synthetase
GSH	Reduced Glutathione
HPA	Hypothalamic-Pituitary Axis
IGF-1	Insulin-like Growth Factor 1
IL6	Interleukin 6
IκB	Inhibitory Factor κ B
KD	Kilodalton
LDH	Lactase Dehydrogenase
MAPK	Mitogen-Activated Protein Kinase
MCA	Middle Cerebral Artery
MEK	MAP/ERK Kinase
MKK	Mitogen-Activated Kinase Kinase
MKKK	Mitogen-Activated Kinase Kinase Kinase

MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy phenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt
MW	Molecular Weight
NFκB	Nuclear Factor κ B
NGF	Nerve Growth Factor
NMDA	N-methyl-D-aspartate
PAM	Octahydro-7-hydroxy-2-methyl-2-phenanthrenemethanol
PACA	Octahydro-7-hydroxy-2-methyl-2-phenanthrenecarboxaldehyde
PACAP	Pituitary Adenylate Cyclase Activating Peptide
PBS	Phosphate Buffered Saline
PKA	Protein Kinase A
PKC	Protein Kinase C
PMS	Phenazine Methosulfate
PROG	Progesterone
RBA	Relative Binding Affinity
RBC	Red Blood Cell
SAR	Structure-Activity Relationship
SF	Serum-free
TGFα	Transforming Growth Factor α

Abstract of Dissertation Presented to the Graduate School
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STRUCTURE ACTIVITY RELATIONSHIP FOR THE NEUROPROTECTIVE EFFECTS OF ESTROGENS: POTENTIAL INVOLVEMENT OF cAMP RESPONSE ELEMENT BINDING PROTEIN AND NUCLEAR FACTOR KAPPA B

By

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Chairman: Joanna Peris
Major Department: Pharmacodynamics

Post-menopausal estrogen replacement therapy is associated with a significant reduction in the incidence of Alzheimer's disease and death due to stroke. Further, estrogens are potent neuroprotective agents in a variety of model systems. The present study has three aims: (1) to elucidate the structural requirements for estratriene neuroprotection; (2) to assess the requirement for a nuclear estrogen receptor (ER) in estratriene neuroprotection; and, (3) to explore an estratriene interaction with the transcription factors CREB and NFκB.

The structure activity relationship (SAR) was determined using a model based on the survival of the human neuroblastoma cell line under conditions of serum deprivation. Addition of physiological concentrations of 17β-estradiol (βE2) attenuated this toxicity. Additionally, the following estratriene compounds were similar to βE2 in neuroprotective

efficacy at a 2 nM dose: 17α-estradiol (αE2), estratrien-3-ol (E-3-ol), 2-hydroxyestradiol, estrone, estriol and 17α-ethynyl estradiol; however, conjugates of these steroids without the phenolic A ring structure were inactive.

We demonstrate the potent neuroprotective ability of estratrienes in a variety of models of neuronal cell types and toxicities including β-amyloid (Aβ) toxicity in SK-N-SH cells and Aβ or excitotoxic insults in rat primary cortical neurons. Of special interest is the protection of the murine hippocampal HT22 cell line by estrogens, as these cells lack functional ERs based upon both binding and reporter gene assays. Treatment with βE2, αE2, or estratriene-3-ol attenuated β-amyloid toxicity in these cells, indicating that estrogen-mediated neuroprotection is not dependent on the presence of a functional ER. Further data supporting this hypothesis are the cytoprotective effects of these compounds on anuclear erythrocytes.

Estrogens can also alter the activity of the transcription factors CREB and NFκB. CREB phosphorylation is increased by estradiol treament, and further, βE2 prevents the serum-deprivation induced decline in CREB. NFκB is a redox sensitive transcription factor whose activity is increased by a number of toxic insults. Concurrent treatment with βE2 or αE2 attenuates the toxin-induced increase in NFκB activity.

In conclusion, the neuroprotective ability of estratrienes requires a phenolic A ring and is not dependent on ER activity. Further, these estratriene compounds modulate the activity of the transcription factors CREB and NFκB. These transcription factors may mediate some effects of estrogens.

CHAPTER 1
INTRODUCTION

The average age at menopause is 54 years, and the current life expectancy for a woman is 78 years. Therefore, most women spend approximately one-third of their lives in an estrogen-deprived state, with this percentage increasing as life expectancy increases. This is important, as estrogen has many beneficial non-reproductive functions, including maintenance of bone mass and improved cardiovascular health. Recent evidence indicates a role for estrogens in normal maintenance of brain function and suggests that the loss of these steroids at menopause may play a role in cognitive decline and neurodegeneration found in Alzheimer's disease (AD). Further, these steroids may play an important role in attenuating neuronal death from acute injuries such as stroke.

Estrogen

17β-estradiol (βE2) is the principal and most potent estrogen secreted by the human ovary. βE2 is synthesized primarily by the ovarian granulosa cells from androstenedione produced by thecal cells. Estrone is formed primarily by extraglandular conversion of androstenedione and to a limited extent βE2 in adipose tissue. βE2 is the predominant estrogen in pre-menopausal women, with plasma levels ranging from about 0.2 nM in the early follicular phase to slightly above 1 nM near the ovulatory surge (Baird and Guevara 1969; Mishell et al. 1971). Plasma estrone concentrations in pre-menopausal women range from about 0.1 to 0.7 nM over the menstrual cycle (Baird and Guevara 1969). With menopause, estrogen levels drop precipitously and βE2 levels average about

0.05 nM (Castelo-Branco et al. 1995). Estrone is the predominant estrogen in post-menopausal women, and plasma concentrations of estrone in post-menopausal women are about 0.08 nM (Castelo-Branco et al. 1995). In men, both estradiol and estrone are formed in extraglandular locations, and plasma levels are usually less than 0.2 nM and 0.3 nM, respectively (Weinstein et al. 1974).

Estrogens are widely used pharmaceutically. Conjugated equine estrogens (CEEs) have consistently been one of the most widely prescribed drugs in the United States (Wysowski et al. 1995). CEEs are a mixture of ten different estrogens purified from the urine of pregnant mares (Bhavnani 1998). The predominant component is estrone sulfate, but equilin compounds (B-ring unsaturated estrogens) are well represented. βE2 and its isomer, 17α-estradiol (αE2) are both present. Common CEE regimens of 1.25 mg/day result in plasma estradiol, estrone, and equilin concentrations of about 0.3 nM, 0.6 nM, and 7 nM, respectively (Whittaker et al. 1980; Castelo-Branco et al. 1995) with equilin concentrations reaching as high as 25 nM approximately 4 h following each dose (Morgan et al. 1979).

Estrogen Receptor (ER): Structure and Regulation

The ER model to explain the mechanism of action for estrogenic hormones was first described in 1968 by two separate laboratories (Gorski et al. 1968; Jensen et al. 1968). The basics of this model remain, although details have changed, as molecular and cell biology techniques have expanded our knowledge of steroid receptor function (reviewed by Tsai and O'Malley 1994).

In this model (Figure 1-1), the steroid estrogen enters the target cells by diffusion and binds to the nuclear ER. The ER is a ligand-activated transcription factor which upon estrogen binding undergoes a series of activation steps (reviewed by Tsai and O'Malley 1994). These steps include a conformational change in the receptor resulting in dissociation of heat shock proteins (HSP), including HSP 90, from receptor complex; formation of receptor dimers; and phosphorylation of the receptor. These changes allow high-affinity binding of the liganded ER to estrogen response elements (ERE), DNA enhancer sites containing the palindromic sequence GGTCANNNTGACC. The activated receptor complex can then interact with basal transcription factors and regulate transcription of the ERE containing genes. Initial effects of estrogens on mRNA levels are seen in 4 h, with maximum stimulation of the mRNA levels requiring days (Harris et al. 1975).

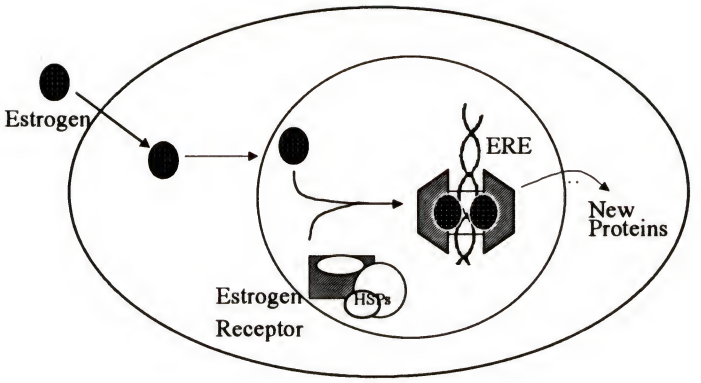


Figure 1-1. A simplified, schematic model of estrogen action at the estrogen receptor. Abbreviations used: HSPs, heat shock proteins; ERE, estrogen responsive element.

The ER is a member of the steroid/thyroid/retinoid receptor gene superfamily (reviewed by Evans 1988). These receptors can be subdivided into functional domains with highly conserved structural features. A schematic representation of the domain structure of the ER is shown in Figure 1-2. The amino terminal A/B domain contains a transactivation domain (AF1), which increases transcriptional activity of the receptor, and

is required for maximal stimulation of some estrogen-responsive genes (Kumar et al. 1987; Tora et al. 1989). The C domain, which is highly conserved among the steroid receptor genes, contains four cysteine residues that form two type II zinc fingers and is responsible for DNA binding (reviewed by Berg 1985). This domain is also involved in receptor dimerization (Evans 1988; Tsai and O'Malley 1994). Domain D, which is highly variable between steroid receptor genes, is thought to function as a hinge region (Evans 1988; Tsai and O'Malley 1994). Domain E is responsible for ligand binding and also contributes to receptor dimerization (Evans 1988; Tsai and O'Malley 1994). A second transactivation domain (AF2), located in the carboxy side of this region, is required for maximal activity (Tora et al. 1989).

The carboxyl terminal region, called the F domain, has no known function, and deletion of this region does not appear to alter ER activity (Kumar et al. 1987).

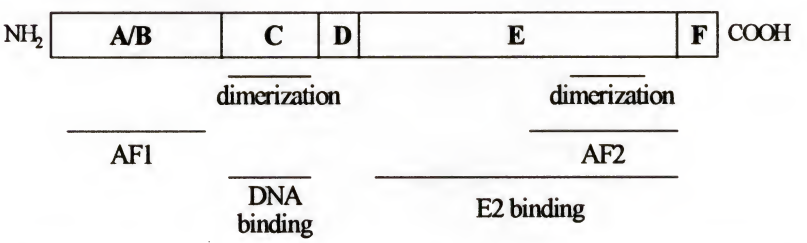


Figure 1-2. A schematic representation of the functional domains of the estrogen receptor. The letter nomenclature of each domain is in boldface and the function is indicated by the lines below.

The ER is a phosphoprotein containing four serine residues and one tyrosine residue which may be phosphorylated. The four serine residues are located in the AF1 region, and phosphorylation of these residues is induced by estrogen binding (Washburn et al. 1991; Ali et al. 1993; Arnold et al. 1994; Le Goff et al. 1994). Mutations which prevent phosphorylation at these sites attenuate ER activity (Ali et al. 1993; Le Goff et al. 1994). The relative contribution of each of the four phospho-serines has been shown to

vary with cell type and promotor (Ali et al. 1993; Arnold et al. 1994; Le Goff et al. 1994). Kuiper and Brinkmann (1994) hypothesize that up to six different protein kinases may be involved in ER phosphorylation. Indeed, multiple signal transduction pathways have been shown to induce this serine phosphorylation and to regulate ER activity, including cyclic adenosine 3',5' monophosphate (cAMP)-protein kinase A (PKA) signaling (Aronica and Katzenellenbogen 1993; Le Goff et al. 1994), protein kinase C (PKC) activity (Le Goff et al. 1994), mitogen-activated protein kinase (MAPK) pathway (Bunone et al. 1996; Joel et al. 1998), and casein kinase (Tzeng and Klinge 1996).

A tyrosine residue in the AF2 region has also been shown to be phosphorylated, although this phosphorylation is not induced by estrogen (Arnold et al. 1995). ER mutations which cannot be phosphorylated at this tyrosine residue lead to constitutive ligand-independent activity of the mutant ER (Zhang et al. 1997). This tyrosine residue has been shown to be a substrate for SRC family kinases (Arnold et al. 1995).

Recently, it has been shown that ER activity is not dependent on the binding of estrogen. This ligand-independent activation of ER can be induced by increased cAMP levels (Ignar-Trowbridge et al. 1996; El-Tanani and Green 1997; Gangolli et al. 1997), phorbol esters (Ignar-Trowbridge et al. 1996), and by a number of growth factors, including IGF-I, EGF, and TGFα (Bunone et al 1996; Ignar-Trowbridge et al. 1996; El-Tanani and Green 1997; Lee et al. 1997; Karas et al. 1998).

Structure Activity Relationship (SAR) for Estrogen-Estrogen Receptor Interactions

βE2 (Figure 1-3), the model ligand for the ER, binds to the ER with a K_d ranging from 0.1 to 1 nM and averaging approximately 0.3 nM (see Sandborne et al. 1971 and

Anstead et al. 1997 for reviews). Estradiol is relatively lipophilic compared to other steroids with a calculated octanol-water partition coefficient of 4.63 (Katzenellenbogen et al. 1982). This lipophilicity is important for ER interactions, as the steroid binds within the hydrophobic core of the receptor. This hydrophobic interaction accounts for more that 50% (an estimated -7.3 kcal/mol of the total binding energy of -13.7 kcal/mol) of the binding energy between βE2 and the ER (Anstead et al. 1997).

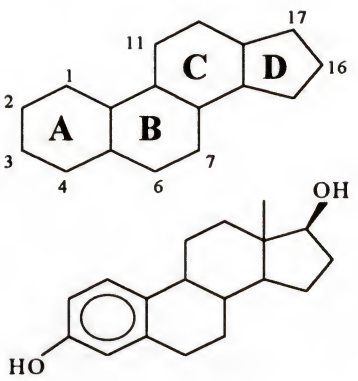


Figure 1-3. A structural representation of the cyclopentaphenanthrene ring (top) and β-estradiol (bottom). Boldface letters indicate ring designation, and the numbers indicate the carbon position in the molecule.

High-affinity ligands for the ER require a phenolic A ring with a hydroxy function at the 3 position of the steroid structure (see Figure 1-3 for number designation of steroid carbons). The aromatic nature of the steroid A ring contributes approximately -1.5 kcal/mol of free energy to the binding interaction whereas the 3-positioned hydroxy function contributes another -1.9 kcal/mol of free energy (Anstead et al. 1997). Relative binding affinity (RBA, βE2 is defined as 100%) is reduced by greater than 95% when the aromatic nature of the A ring is compromised (Zeelen and Bergink 1980; Canceill et al. 1983). Further, removal of the 3-hydroxy moiety reduces RBA by between 92 and 99 % (Chernayaev et al. 1975; Brooks et al. 1987; Anstead et al. 1989; Schwartz and Skafar 1993; Wiese et al. 1997). This 3-hydroxy group of the phenolic A ring is believed to serve as a hydrogen-bond donor (Anstead and Kym 1995) and may be necessary for hydrogen-bond formation with the histidine residue, H524, (Anstead et al. 1997) which has been

shown to be an important contributor in receptor-ligand interactions (Ekena et al. 1996). Alterations that place functional hydrogen-bond acceptors in the 3-position (such as 3-methyl ether or fluorine) remove virtually all receptor binding affinity (Korenman 1969; Katzenellenbogen et al. 1973; Gabbard and Segaloff 1983).

The 17β-hydroxy position also contributes significantly to the affinity of the steroid for the receptor. This 17β-hydroxy contributes an estimated -0.6 kcal/mol of free energy to the steroid-protein binding (Anstead et al. 1997). This group is hypothesized to act as a hydrogen-bond acceptor with a cysteine residue serving as the hydrogen-bond donor (Ikeda 1982). Removal of the 17β-hydroxy group decreases RBA by about 60% (Chernayaev et al. 1975; Brooks et al. 1987; Anstead et al. 1989; Schwartz and Skafar 1993; Wiese et al. 1997). The stereospecificity of the interaction is demonstrated by the low RBA (11-49 %) of the 17α -hydroxy isomer (Korenman 1969; Hahnel et al. 1973; Vander Kurr et al. 1993; Wiese et al. 1997). The importance of the 17β-position is further demonstrated by the binding affinity of the 17 ketone, estrone, which has only a RBA of 11-32% (Korenman 1969; Vander Kurr et al. 1993; Wiese et al. 1997).

The ER is tolerant of non-steroidal ligands such as diethylstilbestrol (DES) (Anstead et al. 1997). Other alterations that are well tolerated include: small to moderate nonpolar groups at the 12β, 16α, and 17α positions (Salman et al. 1986; Fevig et al. 1988; Anstead et al. 1997); nonpolar but sizeable groups at the 7α and 11β positions (French et al. 1993; Belanger et al. 1981); and polar groups far from the ligand core (Anstead et al. 1997).

The estrogenic potency—defined by the ability of the steroid to induce estrogen mediated gene transcription, uterotrophic growth, or MCF-7 breast tumor cell

proliferation— is related to the binding affinity of the compound to the ER, but the relationship is not a direct, linear relationship (Korenman 1969; Wiese et al. 1997). For example, αE2 and estrone have similar binding affinities for the ER but αE2 is 20-fold less potent than estrone in stimulating MCF-7 cell proliferation (Wiese et al. 1997). αE2 is similarly less potent than estrone when estrogenic potency is determined by uterotropic growth stimulation (Korenman 1968). The differences between ER affinity and estrogenic potency may be due to differing affinities of different ligand-ER complexes, with either the ERE or various co-activating proteins necessary for gene-transcription (Clark et al. 1982; Klinge et al. 1992; Kohno et al. 1996; Cheskis 1997).

Estrogen Receptor β

A novel estrogen receptor, called ERβ, was recently cloned from a rat prostate cDNA library (Kuiper et al. 1996). Shortly thereafter, the homologous murine and human ERβ clones were also isolated (Tremblay et al. 1997 and Enmark et al. 1997, respectively). ERβ is not a differential splicing product of the classic ER, now referred to as ERα, but is a different gene product located on chromosome 14 in humans, whereas ERα is located on chromosome 6 (Enmark et al. 1997).

The ERβ transcript shows significant homology to ERα especially in the DNA binding region which has 95 - 97% amino acid sequence homology to the respective ERα in the rat, mouse, and human (Kuiper et al. 1996; Enmark et al. 1997; Tremblay et al. 1997). Similarly, the ligand binding region shows 55 - 60% homology in all three species. ERα and ERβ show no difference in binding affinity for βE2 with K_d values for both receptors between 0.1 to 0.5 nM of βE2 (Kuiper et al. 1997; Tremblay et al. 1997;

Witkowska et al. 1997). Only minimal differences, if any, are seen in binding affinity between the two receptors with a large variety of steroid ligands (Kuiper et al. 1996; Kuiper et al. 1997; Tong et al. 1997; Tremblay et al. 1997). Indeed, only 16-iodo-estradiol and genestin have been shown to differentiate between the two receptors (Kuiper et al.1997; Witkowska et al. 1997; Tong et al. 1997).

ERβ forms both homodimers and heterodimers with ERα and specifically binds to EREs with essentially the same *in vitro* DNA binding characteristics (Pace et al. 1997; Tremblay et al. 1997). Both receptors have similar trans-activation properties on ERE-containing genes, with ERα showing a slightly higher level of induction in reporter gene assays (Kuiper et al. 1996; Tremblay et al. 1997). Kuiper et al. (1996) have reported a slight increase in constitutive transcriptional activity with the rat ERβ that has not been seen with the murine ERβ (Tremblay et al. 1997). Interestingly, ERα and ERβ have opposing effects on AP1-mediated transcription (Paech et al. 1997). βE2 activates AP1-mediated transcription in the presence of ERα but is inhibitory in the presence of ERβ. However, the anti-estrogens, tamoxifen, raloxifene, and ICI 164384, increase AP1-mediated transcription in the presence of either ER.

ERβ is also a phosphoprotein and appears to be regulated by phosphorylation at both serine and tyrosine residues. The AF1 region has potential extracellular regulated kinase (ERK) phosphorylation sites (Tremblay et al. 1997). Increased activity of the Ras-Raf-MAPK pathway by expression of a dominant active Ras significantly enhances βE2-mediated transcriptional activity of ERβ (Tremblay et al. 1997). Mutation of one of the potential ERK phoshorylation sites abrogated the effect of the dominant active Ras expression on ERβ-mediated activity. ERβ also contains a tyrosine residue that is a

potential phosphorylation site (Tremblay et al. 1998). Mutation of this tyrosine residue to abolish phosphorylation results in constitutive activation of the ERβ receptor.

Estrogen and Estrogen Receptors in the Brain

Estrogens may be synthesized and/or metabolized by the brain (reviewed by Zhu and Conney 1998). Aromatase, which converts androgens to estrogens, is localized in many brain regions, and the resulting estrogens have been shown to be important in mediating many androgenic effects on the brain (reviewed by Naftolin and MacLusky 1982). βE2 is locally metabolized by 2-hydroxylation into catecholestrogens which serve to modulate catecholamine responses (Fishman and Norton 1975). One study indicates that nM concentrations of catecholestrogens are present in the cortex of intact female rats (Ball and Knuppen 1980). A study by Rahimy et al. (1990) found that 24 h following a single injection of βE2, the concentrations in the brain were approximately 10-fold higher than in the plasma suggesting that brain concentrations of the steroid may normally exceed plasma levels.

Both ERα and ERβ are found throughout the rat brain, though, overall, ERβ is more abundant and has a wider distribution (Kuiper et al. 1997; Österlund et al. 1998; Shughrue et al. 1997). Differential expression of the two ERs have been seen in rat hypothalamic nuclei (Shughrue et al. 1997; Österlund et al. 1998). ERα expression appears strong in the preoptic area, arcuate nucleus, and ventromedial nucleus, whereas ERβ expression has been seen in the preoptic area, supraoptic nucleus, and ventromedial nucleus. Both ERs have been shown to be expressed in the rat cortex and hippocampus, although ERα expression appears very weak (Shughrue et al. 1997). In primates, both

ERα and ERβ expression has been seen in the hippocampus and hypothalamus, and again, the ratio of ERβ to ERα expression is relatively high (Register et al. 1998).

Estrogen and Neuronal Signal Transduction

The classical mechanism of estrogen action is through binding to nuclear estrogen receptors followed by transcription mediated by binding of the steroid-ER complex to the ERE of estrogen-response genes. It is becoming clear that this mechanism is insufficient to explain many of the effects of estrogen. Sukovich et al. (1994) have demonstrated that estrogen may regulate expression of non-ERE-containing genes. Further, many effects of estrogens occur too rapidly (seconds to minutes) to require ER-mediated gene transcription (Garcia-Segura et al. 1987; Wong and Moss 1991, 1992; Briton 1993).

In non-neuronal tissues, estradiol has been shown to interact with the MAPK pathway (Ignar-Trowbridge et al. 1992; Migliacco et al. 1996), increase cAMP levels (Szego and Davis 1967; Aronica et al. 1994), and modulate calcium fluxes (Morley et al. 1992). Increasingly, interactions of estrogens with intracellular signal transduction pathways (Gu et al. 1996; Gu and Moss 1996; Zhou et al. 1996; Watters et al. 1997; Watters and Dorsa 1997; Singh et al. 1999) and effects of estrogens on membrane excitability (Dorner et al. 1980; Wong and Moss 1991) are being described in neuronal cells.

Cyclic AMP-protein kinase A-cAMP response element binding protein pathway

The cAMP-PKA-cAMP response element binding protein (CREB) pathway is a well-described signal transduction pathway which is modulated by G-protein coupled receptors (reviewed by Lee and Masson 1993; Papavassiliou 1994). Activation of

adenylate cyclases (AC) directly increase intracellular concentrations of cAMP which then activates PKA by binding to the two regulatory sites on the enzyme. The active kinase then phosphorylates a number of proteins including the nuclear transcription factors CREB (Figure 1-4). Phosphorylation on the serine 133 residue dramatically enhances the transactivating capabilities of CREB and the subsequent transcription of cAMP response element (CRE)-regulated genes.

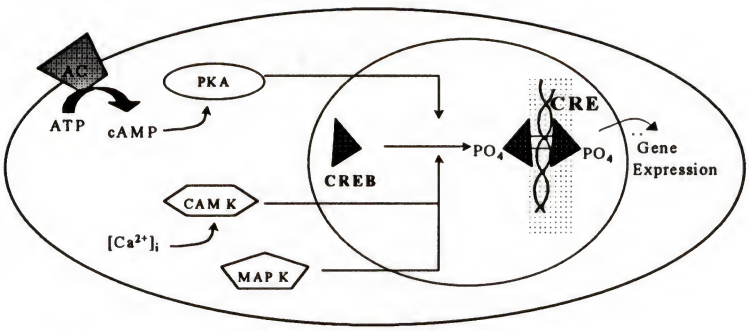


Figure 1-4. A simplified, schematic representation of major pathways involved in the CREB phosphorylation including the cAMP-PKA pathway. Abbreviations used: AC, adenylate cyclase; PKA, protein kinase A; CAMK, Ca^{2+}-Calmodulin-dependent kinase; MAPK, mitogen-activated protein kinase; CRE, cyclic AMP response element; CREB, CRE binding protein.

Estradiol treatment has been shown to increase cAMP accumulation in both rat hypothalmic neurons (Gunaga et al. 1974; Weissman et al. 1975) and human neuroblastoma cells (Watters and Dorsa 1998). In all three studies, initial effects of βE2 were seen at 30 to 50 min, and cAMP levels returned to baseline by 90 min. This effect of βE2 required doses of the steroid 30- to 1000-fold greater than the concentration required for activation of the ER.

βE2 treatment has been shown to have effects consistent with an increase in PKA activity. In hippocampal neurons, 10 nM of βE2 potentiated kainate-induced currents in a manner similar to 8-bromo-cAMP (Gu and Moss 1996). This effect of βE2 was inhibited by the two different PKA inhibitors (Gu and Moss 1996). Lagrange et al. (1997) have

demonstrated that βE2 (1 to 20 nM) modulates agonist activation of μ-opioid receptors. This effect is mimicked by cAMP analogues and is inhibited by PKA inhibitors and the anti-estrogen, ICI 164-384, but not by the protein synthesis inhibitor cyclohexamide.

βE2 exposure also increases the phosphorylation of CREB on the serine 133 residue in hypothalamic neurons (Gu et al. 1996; Zhou et. al. 1996) and in a human neuroblastoma cell line (Watters and Dorsa 1998). Consistent with the effect of βE2 on cAMP accumulation, this effect required at least 1 to 10 nM βE2, and with the exception of one study (Zhou et al. 1996) which demonstrated increased levels of phosphorylated CREB (PO_4-CREB) immunoreactivity by 15 min, followed a similar time course with evidence of increased CREB phosphorylation seen 30 min to 90 min after βE2 treatment (Gu et al. 1996; Watters and Dorsa 1998). Partial estrogen antagonists blocked the βE2-induced increase in PO_4-CREB immunoreactivity in one study (Nafoxidine; Gu et al. 1996) but not in another (Tamoxifen; Watters and Dorsa 1998). βE2 treatment did not alter total CREB immunoreactivity (Gu et al. 1996; Zhou et. al. 1996; Watters and Dorsa 1998). It should be noted that CREB can be phosphorylated by pathways other than cAMP-PKA (see Figure 1-4; Sheng et al. 1990; Dash et al. 1991; Sheng et al. 1991; Xing et al. 1998), and these pathways, the MAPK pathway and the CAMK pathway, have also been shown to be activated by βE2 exposure (Singh et al. 1999; Hayashi et al. 1994, respectively).

The doses of βE2 required for activation of the cAMP-PKA-CREB pathway, the time-course for the effects and the mixed effect of anti-estrogens suggest that this effect is not mediated by a classical ER pathway but it is unclear whether or not either known ER is involved. There is some suggestion that a membrane ER exists (Pietras and Szego

1979; Pappas et al 1995; Moss et al. 1997; Razandi et al. 1999) and speculation that this proposed membrane ER may be G-protein coupled (Moss et al. 1997; Razandi et al. 1999).

Mitogen activated protein kinase pathway

The MAPK pathways in mammalian cells consist of a series of kinases in which each is successively activated by phosphorylation (reviewed by Cano and Mahadevan 1995; Brunet and Pouyssegur 1997). In the archetypal model (see Figure 1-5), MAP kinase kinase kinase (MKKK) activates MAP kinase kinase (MKK) by phosphorylation on two serine/threonine residues. The active MKK is a dual specificity kinase which then phosphorylates MAPK on both a threonine and a tyrosine residue. The active MAPK is a proline-directed serine/threonine kinase and phosphorylates a variety of other kinases and transcription factors.

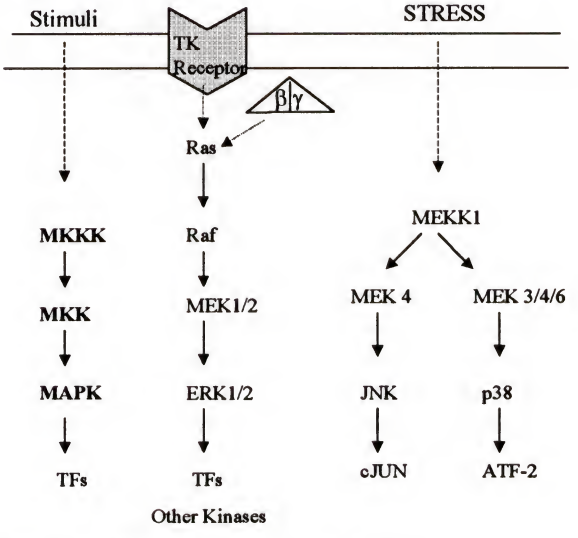


Figure 1-5. A simplified, schematic representation of the MAP kinase pathways. The more general nomenclature shown in bold. Abbreviations used: TK, tyrosine kinase receptor; TF, transcription factor; MAPK, mitogen-activated protein kinase; MKK, MAPK kinase; MKKK, MKK kinase; ERK, extracellular-signal regulated kinase; MEK, MAP/ERK kinase; MEKK, MEK kinase.

Parallel MAPK pathways exist. The JNK and p38 pathways are activated by cytokines and environmental stress, and these kinases are involved in apoptosis and stress responses. Activation of the ERK pathway is generally associated with cell growth,

differentiation, and protection, and is activated by a vast array of peptide growth factors including the neurotrophins (reviewed by Cano and Mahadevan 1995; Brunet and Pouyssegur 1997).

As mentioned previously, peptide growth factors signaling through the ras-raf-MEK-ERK pathway regulate both estrogen-dependent and estrogen-independent ER activity (Bunone et al 1996; Ignar-Trowbridge et al. 1996; El-Tanani and Green 1997; Lee et al. 1997; Karas et al. 1998). In neurons, the expression of the neurotrophin receptor trk A as well as the neurotrophins, nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) is increased by estrogen (Sohrabji et al. 1994; Singh et al. 1995; Sohrabji et al. 1995b; Miranda et al. 1996). Similarly, NGF exposure increases expression of the ER (Sohrabji et al. 1994; Miranda et al. 1996). Similar to neurotrophins, βE2 has been shown to have neurite-promoting effects (Toran-Allerand 1976, 1980; Ferreira and Caceres 1991; Woolley and McEwen 1992; Brinton 1993).

Recently, it has been demonstrated that estradiol can rapidly activate the ras-raf-MEK-ERK pathway in neuroblastoma cells and cortical explants (Watters et al. 1997; Singh et al. 1999). Phosphorylation of both ERK1 and ERK2 was seen within 5 to 15 min of βE2 exposure with no change in the immunoreactivity of total ERK1 and ERK2. Singh et al. (1999) hypothesize that a multimeric complex consisting of the ER and b-raf mediates the increase in ERK phosphorylation. In support of this, they have demonstrated that βE2 treatment results in a rapid increase in b-raf activity that co-immunoprecipitates with the ER.

Antioxidant Properties of Estrogen

The lipophilic phenolic structure of estrogenic steroids suggest that they possess antioxidant activity particularly relevant in attenuation of lipid peroxidation cascades (Niki 1987). In both cell-free and cell culture systems, estrogens have been shown to be potent inhibitors of lipid peroxidation effective at low μM concentrations (Nakano et al. 1987; Sugioka et al. 1987; Mukai et al. 1990; Hall et al. 1991; Mooradian 1993; Lacort et al. 1995; Goodman et al. 1996). The potency of estrogens in these systems is similar to the antioxidant potency of α-tocopherol tested under the same conditions (Nakano et al. 1987; Sugioka et al. 1987; Mukai et al. 1990; Hall et al. 1991). Estrogens have also been shown to inhibit peroxide formation induced by a variety of oxidative stresses in a clonal hippocampal cell line (Behl et al. 1997b). These antioxidant properties of estrogens have been shown to be dependent upon the presence of a phenolic A ring in the steroid structure (Nakano et al. 1987; Sugioka et al. 1987; Mukai et al. 1990; Lacort et al. 1995; Behl et al. 1997b).

Neuronal Death and Neurodegeneration

β-Amyloid Toxicity

β-Amyloid peptide (Aβ) is a hydrophobic, non-glycosylated peptide of 39 to 43 amino acids generated from amyloid precursor protein (APP; reviewed by Selkoe 1994). Aβ is capable of self-aggregation into amyloid fibrils which form a core component of senile plaques, the hallmark of post-mortem AD diagnosis (reviewed by Selkoe 1994).

Exposure of cultured neurons to Aβ results in a profound disruption of cellular morphology (Yanker et al. 1990) and cell death (Yanker et al. 1989; Behl et al. 1992;

Mattson et al. 1993). Aβ exposure leads to calcium ion dyshomeostasis (Mattson et al. 1993) and has been reported to sensitize neurons to glutamate toxicity (Koh et al. 1990). Aβ toxicity is associated with increased oxidative stress, including generation of intracellular peroxides (Behl et al. 1994), and increases in lipid peroxidation (Behl et al. 1992; Goodman et al. 1996). It is a model for the amyloid-load observed in AD.

Glutamate Toxicity

L-glutamate, the major excitatory amino acid (EAA) neurotransmitter in the brain, is involved in many neurologic functions such as cognition and memory; however, glutamate neurotoxicity, a type of excitotoxicity, may mediate neuronal death in several pathological conditions, including stroke, epilepsy, Huntington's disease, and AD (reviewed by Lipton and Rosenberg 1994). Glutamate-induced excitotoxicity is a multi-faceted process that includes excessive influx of sodium and calcium ions into neurons through α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)/kainate and/or N-methyl-D-aspartate (NMDA) channels (Choi 1992). The resulting high concentrations of intracellular calcium lead to a cascade of toxic events including production of free radicals.

Serum-deprivation Toxicity

Serum deprivation of cultured neuronal cells results in either differentiation of the cells (Howard et al. 1993) or apoptosis (Batistato and Greene 1991, 1993; Galli and Fratelli 1993; Mesner et al. 1995; Satoh et al. 1996; Haviv et al. 1998; Korhonen et al. 1998; Wadia et al. 1998; Yano et al. 1998). Serum-deprivation-induced apoptosis is well characterized and involves a decrease in mitochondrial membrane potential and associated mitochondrial ion dyshomeostasis (Wasia et al. 1998), activation of the pro-apoptotic

protein BAD (Yano et al. 1998), activation of capases (Haviv et al. 1998; Korhonen et al. 1998), and endonuclease cleavage of DNA (Batistato and Greene 1991, 1993). Serum deprivation leads to a rapid decline in ras activity (Mesner et al. 1995), and the associated toxicity is prevented by growth factors which increase ras activity (Batistato and Green 1991, 1993). Serum-deprivation toxicity may involve oxidative stress, as intracellular levels of peroxyl radicals increase with serum-deprivation. Additionally, antioxidant compounds can attenuate serum-deprivation-induced neuronal death. Serum deprivation of the human neuroblastoma SK-N-SH cell line is used extensively in this dissertation, as βE2-induced neuroprotection has been previously demonstrated in this model.

cAMP and Neuronal Death

Several studies correlate increased intracellular cAMP concentrations with prevention of neuronal apoptosis. Treatment with cAMP analogues or forskolin, an AC activator, promotes long term survival of cerebellar granule neurons (D'Mello et al. 1993) and spinal motor neurons (Hanson et al. 1998) in culture. Treatment with pituitary adenylate cyclase activating peptide (PACAP) is associated with an increase in cAMP accumulation and similarly increases cerebellar granule neuron survival (Campard et al. 1997). The beneficial effect of PACAP is attenuated by expression of a dominant inhibitory PKA mutant. cAMP analogues can also increase survival and promote neurite outgrowth of primary rat sympathetic neurons in culture (Rydel and Greene 1988). This protective effect is blocked by competitive cAMP antagonists. Increased intracellular cAMP can also protect septal cholinergic neurons from apoptosis induced by NGF withdrawal (Kew et al. 1996) and rat PC12 cells from sialoglycopeptide exposure

(Kobayashi and Shinozawa 1997). Goswami et al. (1998) have shown that opiate-induced apoptosis in a mouse neuroblastoma cell line is associated with a decrease in cAMP concentrations, and that toxicity is blocked by cAMP agonists and enhanced by cAMP antagonists.

Two studies have suggested that CREB may play an important role in neuroprotection. Walton et al. (1997), using an hypoxic-ischemic injury model, demonstrated a decline in PO_4-CREB immunoreactivity in CA1 pyrimidal cells which do not survive mild hypoxia-ischemia injury. In contrast, the more resistant dentate granule cells and cortical cells showed an increase in PO_4- CREB immunoreactivity. Hypoglycemic seizure selectively reduces CREB immunoreactivity (Panickar et al. 1997). This decline in CREB immunoreactivity does not appear to be due to cell loss, as the rats were sacrificed at a time point prior to cell death (90 min post-seizure). Interestingly, however, the regions of CREB decline correlated with regions that have previously been shown to have massive cell loss at one week following hypoglycemic seizure (Auer et al. 1985). Therefore, the decline in CREB levels may be leading to cell loss.

Nuclear Factor κB and Neuronal Death

Nuclear factor κB (NFκB) is a redox sensitive transcription factor composed of a p50 and p65 subunit that was originally identified as a

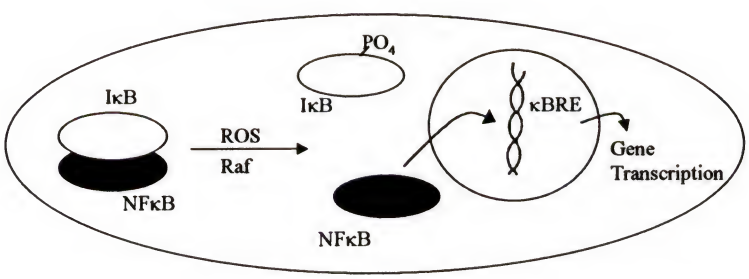


Figure 1-6. A schematic representation of NFκB activation. Abbreviations used: NFκB, nuclear factor κ B; IκB, inhibitory factor κ B; ROS, reactive oxygen species, κBRE, κ B response element.

regulator of immunogloblin κ light chain expression in B cells (reviewed by Verma et al. 1995). NFκB is sequestered in the cytosol by a non-covalent interaction with its inhibitory protein, inhibitory factor κ B (IκB). IκB is phosphorylated by raf kinase and also in response to the presence of reactive oxygen species. Phosphorylation or phosphorylation coupled with degradation of IκB reveals the nuclear localization sequence of NFκB resulting in NFκB activation (Baeuerle and Baltimore 1988).

The role of NFκB in cell survival is complex, as NFκB activation is implicated in both cell death promotion and cell protection (see Lipton 1997 for review). NFκB activity is increased by a number of toxic insults, including ischemia (Clemens et al. 1997), glutamate exposure (Kaltschmidt et al. 1995), Aβ exposure (Lezoualc'h and Behl 1997; Kaltschmidt et al. 1997), and H_2O_2 exposure (Schreck et al. 1991; Schmidt et al. 1995; Meyer et al. 1993; Lezoualc'h and Behl 1997). Significantly, many antioxidant compounds that protect neurons from these oxidative stress-induced deaths also inhibit the toxin-induced increase in NFκB activity (Meyer et al. 1993; Grilli et al. 1996; Lezoualc'h et al. 1998a; Post et al. 1998). NFκB leads to transcription of genes involved in cellular defense as well as genes that are involved in apoptosis. For example, NFκB activation increases expression of γ-glutamylcysteine synthetase (γGCS) and concurrently, the intracellular level of reduced glutathione (GSH; Lezoualc'h et al. 1998b) which increases the overall antioxidant defenses of the cell. Further, NFκB also regulates expression of several pro-apoptotic proteins, including p53 (Wu and Lozano 1994), a potent initiator of apoptotic death and interleukin-1-converting enzyme (ICE; Casano et al. 1994).

Of specific interest to AD, both Aβ (Schreck et al. 1991; Kaltschmidt et al. 1997) and the paired helical filament tau (Yan et al. 1995) increases NFκB activity. Further, the

APP gene has two NFκB binding domains in its enhancer region (Grilli et al. 1995), and increased NFκB activity is correlated with an increase in Aβ secretion (Yan et al. 1995). Other genes regulated by NFκB that are of specific interest to the pathophysiology of AD include interleukin 6 (IL6; Ray et al. 1988), a pro-inflammatory cytokine found in high concentration in senile plaques, and α_1-antichymotrypsin (Lieb et al. 1996), a protease inhibitor found in senile plaques bound to Aβ protein (Rozemuller et al. 1990) and potentially involved in the Aβ fibril formation (Vandenabeele and Fiers 1991).

Estrogen and Neuroprotection

Women, Estrogen, and the Risk of Alzheimer's Disease

Age-matched epidemiological studies reveal that women are twice as likely as men to develop AD. This observation is irrespective of geographical location, as studies in the U.S. (Aronson et al. 1990), Europe (Rocca et al. 1991), Japan (Rocca et al. 1986), and China (Zhang et al. 1990) find an increased prevalence of AD among women. Further, Aronson et al. (1990) found a significant interaction between gender and history of myocardial infarction in the risk of AD. Women with a history of myocardial infarction were five times more prone to AD than other women while a history of myocardial infarction had no effect on the risk of men.

Other epidemiological studies have examined the role of estrogen in the risk of AD in women. Henderson et al. found that AD patients were less likely to use estrogen replacement therapy (ERT) than controls, although the groups did not differ in age or the total number of prescribed medications (Henderson et al. 1994). Furthermore, the AD patients who were estrogen users showed a significantly better cognitive performance as

measured by the Mini-Mental State examination that was not due to differences in the age, education, or symptom duration between estrogen users and non-users. A retrospective study by Paganini-Hill and Henderson found not only that estrogen use reduced the risk of AD and related senile dementia diagnoses, but that this effect is both dose and duration dependent (Paganini-Hill and Henderson 1994). Two similar case-control studies by Mortel and Meyer (1995) and Warring et al. (1997) also found that ERT was associated with a decreased risk of AD (odds ratios of 0.6 with a 95% confidence interval of 0.3 to 1.2, and 0.4 with a 95% confidence interval of 0.2 to 0.8, respectively). One study found no significant correlation between current and former estrogen use and the incidence of AD (Brenner et al. 1994). However, the odds ratio of current estrogen use in the risk of AD in this study (0.6 with a 95% confidence interval of 0.3 to 1.2) was similar to that found by Paganini-Hill and Henderson (0.7 with a 95% confidence interval of 0.4 to 1.2) suggesting that a trend toward decreased risk with ERT was observed. Prospective cohort studies have found a 50 % reduction in the risk of AD associated with ERT (Kawas et al. 1997; Tang et al. 1996). Further, this reduction in risk was seen irrespective of ethnic background or apolipoprotein E allotype.

Estrogen and Alzheimer's Disease: Clinical Trials

Several small clinical trials have examined the effects of estrogen therapy on cognition and memory in AD patients. Fillit et al. (1986) treated seven women exhibiting Alzheimer's type dementia with estradiol for a six week period. Scores on a variety of psychometric tests were evaluated prior to estrogen treatment and at 3 and 6 weeks of treatment. Three of the women responded to the estrogen therapy with improved scores

on three of the psychometric tests. The scores returned to baseline as serum estradiol levels declined in the post-treatment period. The scores of the other women were not different from baseline at either time point. The estrogen responsive group differed from the non-responsive group with an older age of dementia onset and less severe dementia. A higher rate of osteoporosis and lower post-treatment serum estradiol levels was also found in the responder group suggesting that an estrogen-deficiency may contribute to dementia in some women.

Similarly, Honjo et al. (1989) found that 6 weeks of estrogen therapy improved the cognition of women with AD. This improvement was not due to practice effects, as the scores of untreated women with AD did not change. Furthermore, the pre-treatment serum estrogen levels of women with AD were lower than for age-matched non-demented controls suggesting that low serum estrogen levels could contribute to Alzheimer's type dementia. Low-dose (0.625 mg CEE per day), long-term estrogen therapy has also been shown to be effective in preventing the cognitive decline of AD patients (Ohkura et al. 1994b). Over the study period, the cognitive performance of untreated AD patients declined while those of patients receiving low-dose estrogen treatment improved or remained constant. The mean scores between groups were not significantly different prior to treatment on either the Mini-Mental State Examination or the Hasegawa Dementia Scale. At 5 months, the scores on both tests were significantly higher in the estrogen group. Nearly identical results were seen with a similar study design but with shorter term (5 to 6 weeks) and higher dose (1.25 mg CEE per day) of estrogen use (Honjo et al. 1989; Ohkura et al. 1994a).

ERT may also enhance the beneficial effects of cholinesterase inhibitors on cognition. Schneider et al. (1996) found that women taking both the cholinesterase inhibitor, Tacrine, and oral estrogens performed significantly better on measures of cognitive performance than did women taking either treatment alone.

Epidemiology of Stroke and Estrogen Therapy

Stroke is the third leading cause of death in the United States, behind heart disease and cancer, and a major contributor to disability (Feinleib et al. 1993). A 50-year-old, white woman has a 20% lifetime risk of having a stroke and an 8% likelihood of dying from a stoke (Grady et al. 1992). The literature on the effect of ERT on the incidence of stroke is conflicting. Several studies suggest significant reduction in incidence of stroke with estrogen use (Falkeborn et al. 1993; Finucane et al. 1993; Lidnenstrøm et al. 1993; Lafferty and Fiske 1994) while others find no correlation between incidence of stroke and ERT (Boysen et al. 1988; Stampfer et al. 1991; Grodstein et al. 1996; Pedersen et al. 1997; Petitti et al. 1998). One study even found a significantly increased risk of stroke with estrogen use (Wilson et al. 1985); however, these results were statistically adjusted for lipid profile. This confounds interpretation of the data, as ERT has beneficial effects on the lipid profile, and this effect may mediate a reduction in risk for stroke. ERT had no effect on the risk of stroke when therapy was began after diagnosis with coronary disease (Hulley et al. 1998). Stroke is a group of clinically heterogeneous diseases which include cerebral infarctions (thrombosis and embolism), intracerebral hemorrhage, and subarachnoid hemorrhage. While risk factors, including susceptibility after ERT, may differ for different stroke entities (reviewed by Paganini-Hill 1995a), only three of these

studies (Wilson et al. 1985; Stampfer et al. 1991; Falkeborn et al. 1993) differentiated between stroke entities. Two of these studies (Stampfer et al. 1991; Falkeborn et al. 1993) report differing relative risks of ERT for different stoke entities.

While the effect of estrogen on stroke incidence is unclear, studies consistently indicate a protective role of ERT on stroke-related mortality. Estrogen therapy is associated with a 40 to 60% reduction in death from stroke and other cerebral vascular diseases (Bush et al. 1987; Petitti et al. 1987; Paganini-Hill et al. 1988; Hunt et al. 1990; Henderson et al. 1991; Finucane et al. 1993). Schmidt et al. (1996) found ERT was associated with a lower frequency and lower extent of white matter abnormalities representative of "silent" ischemic damage. Together, these studies suggest that estrogens may have a role in attenuation of brain injury associated with stroke.

Estrogen and Middle Cerebral Artery Occlusion in Animal Models

Recent evidence indicates sex differences in brain damage following experimental ischemic stroke. Hall et al. (1991) reported less mortality, less neuronal pathology, more rapid recovery of calcium homeostasis, and decreased oxidative stress in female gerbils as compared to their male counterparts after a period of incomplete ischemia. The gender difference was not due to improved cerebral blood flow (CBF), as no differences between males and females were seen in CBF before, during or immediately after ischemia. Female rats have been reported to have smaller infarct volumes (Alkayed et al. 1998; Zhang et al. 1998). Further, ovariectomy of the female rats resulted in infarct sizes comparable to male rats. In contrast to the findings of Hall et al. (1991) in gerbils, Alkayed et al. (1998) found higher baseline CBF values and less reduction in CBF with ischemia in intact female rats.

This end-ischemic difference in blood flow was seen only in the caudate-putamen, not the cortex although cortical tissue was protected.

In ovariectomized female rats, estrogen replacement can decrease mortality from middle cerebral artery occlusion (MCAO; Simpkins et al. 1997; Zhang et al. 1998) and significantly reduce the volume of the infarct (Shi et al. 1997; Simpkins et al. 1997; Dubal et al. 1998; Zhang et al. 1998). This protection is seen with physiologically relevant doses of estradiol administered one day to one week prior to either transient or permanent MCAO (Simpkins et al. 1997; Dubal et al. 1998). A single injection of βE2 administered up to 90 minutes post-occlusion is still effective at reducing the infarct size (Simpkins et al. 1997). The protection conferred by estrogens appears to be regionally specific to the cortex, as only minimal reduction of striatal lesion volume is seen (Dubal et al. 1998; Simpkins, J.W. and Shi, J., unpublished observations). βE2-treatment also reduces lesion size in castrate and intact male rats (Hawk et al. 1998; Toung et al. 1998).

The effect of estrogen on infarct volume is not attributable to increased blood flow, since no effect of estrogen on CBF has been seen during ischemia (Dubal et al. 1998; Stubley et al. 1998; Toung et al. 1998). However, βE2 treatment appears to affect the rate at which CBF normalizes post-occlusion (Stubley et al. 1998).

Neuroprotective Effects of Estrogens in Culture

Our laboratory first demonstrated that physiological doses of the potent estrogen, βE2, could exert direct cytoprotective effects on a neuronal cell line using a human neuroblastoma cell line, SK-N-SH, under the conditions of serum deprivation (Bishop and Simpkins 1994). This is a cytoprotective effect of βE2 rather that a mitogenic effect, as

treatment with βE2 does not increase ^{3}H-thymidine uptake in these cells (Bishop and Simpkins 1994). Additionally, βE2 can attenuate oxidative stress-induced toxicity such as exposure of neurons to the β-amyloid peptide (Aβ; Behl et al. 1995; Goodman et al. 1996; Green et al. 1996) or exposure to H_2O_2 (Behl et al. 1995). βE2 treatment can decrease cell death in rat primary neurons due to excitotoxic insults such as EAA exposure (Behl et al. 1995; Goodman et al. 1996; Singer et al. 1996; Zaulynov et al. 1999) and anoxia/ reoxygenation (Zaulynov et al. 1999).

Objectives

The mechanism by which estrogens exert their neuroprotective actions is unclear. Possible mechanisms include estrogen-induced expression of protective proteins through a classical nuclear estrogen receptor, antioxidant properties of the steroid, or involvement in neuroprotective signal transduction cascades. The goal of this dissertation is to test the hypothesis that the classical nuclear ER pathway as shown is Figure 1-1 is required for the neuroprotective effects of estrogens. This hypothesis will be accomplished by (1) determining if the SAR for estrogens neuroprotective effects corresponds to the SAR for classical ER-mediated effects; (2) determining if neuronal cells can be protected by estrogens in the absence of the classical nuclear ER; and, (3) determining if the estratrienes can protect red blood cells (RBCs) which lack a nucleus, and therefore cannot utilize classical ER-mediated pathway. Further, the hypothesis that estrogens interact with the transcripton factors CREB and NFκB will be tested.

CHAPTER 2
GENERAL METHODS

Cell Culture

Culturing of Cell Lines

SK-N-SH cells

SK-N-SH neuroblastoma cells were obtained from American Type Tissue Collection (Rockville, MD) and were grown to confluency in RPMI-1640 media supplemented with 10% fetal bovine serum (FBS; Sigma Chemical Co, St. Louis, MO or Hyclone, Logan, UT), 100 U/ml penicillin G, and 100 µg/ml streptomycin (all reagents from Sigma Chemical Co. or GIBCO, Gaithersburg, MD) in monolayers in plastic Nunc 75 cm^2 flasks (Fisher Scientific, Orlando, FL) at 37°C and under 5% CO_2/95% air. Medium was changed three times weekly. Cells were observed with a phase-contrast microscope (Nikon Diaphot-300). SK-N-SH cells were back-cultured every 5-7 d using standard trypsinization procedures to maintain the cell line. SK-N-SH cells were used in passes 32-45. The media itself does not contain estrogens and the FBS does not contain detectable levels of estrogens (detection limit = 10 pM).

HT-22 cells

HT-22 cells, a murine neuronal cell line of hippocampal origin, were the generous gift of Dr. David Schubert (Salk Institute, San Diego, CA). The cells were maintained as described for SK-N-SH cells with the exception that for indicated studies, cells were maintained in DMEM media (GIBCO) supplemented with 10% charcoal stripped FBS,

100 U/ml penicillin G, and 100 µg/ml streptomycin. HT-22 cells were used within 33 passes of receipt.

MCF-7 cells

MCF-7 cells, a human breast tumor cell line, were obtained from American Type Tissue Collection (Rockville, MD) and were maintained as described for SK-N-SH cells with two exceptions. Cells were grown in DMEM media (GIBCO) supplemented with 10% charcoal stripped FBS, 2.5 µg/ml bovine insulin, 100 U/ml penicillin G, and 100 µg/ml streptomycin. Cells were passed every 10-14 days. MCF-7 cells were changed into phenol-red free media for 48 h prior to all experiments, and all experiments on this cell type were carried out in phenol-red free media. MCF-7 cells were used in passes 134-160.

Primary rat cortical neurons

Cultures of primary rat cortical neurons were supplied by Dr. Colin Sumner's laboratory (University of Florida, Gainesville, FL) by methods previously described (Chandler et al. 1993). Briefly, cortical tissue from 1 d old Sprague-Dawley (Charles River Farms, Wilmington, MA) was separated from blood vessels and pia mater and sectioned into approximately 2-mm chunks. Tissue was then trypsinized in an isotonic salt solution (pH 7.4) at 37°C. Cells were treated with DNAse I in the presence of DMEM media containing 10% plasma-derived horse serum (Central Biomedia, Irwin, MO) and triturated. The cell suspensions were centrifuged, and the resulting cell pellet was suspended in DMEM with 10% plasma-derived horse serum on 35 mm culture dishes pre-coated with poly-l-lysine at a density of 4×10^6 cells per dish. Cells were then incubated

in a humidified incubator at 37°C with 10% CO_2/90% air. Cells were exposed to β-cytosine arabinoside on days 3 and 4. Neurons were used 9-12 days *in vitro*. At this time, cultures generally consist of approximately 90% neurons (Chandler et al. 1993).

Toxin Exposure

Aβ peptide

Aβ amino acids 25-35 (Bachem, Torrance, CA) was initially dissolved 1 mg in 200 µl sterile dH_2O. PBS (800 µl) was then added. This solution was then diluted to the final concentration in the experimental media. Aβ amino acids 1-40 (Bachem) was prepared similarly to the Aβ (25-35) but was incubated at 37°C for 4 d prior to use as required for aggregation (Pike et al. 1993). Cells were exposed to Aβ for time points ranging from 2 to 4 d at concentrations ranging from 1 to 20 µM.

Hydrogen peroxide

H_2O_2 (30% v/v, stabilized; Sigma Chemical Co.) was diluted to a 300 mM concentration in sterile water, and then diluted to the final concentration in the experimental media. H_2O_2 was used at concentrations ranging from 1 µM to 300 µM.

Excitatory amino acids

NMDA, AMPA, and glutamate (Sigma Chemical Co.) were dissolved initially in sterile dH_2O at a concentration of 1-10 mg/ml and diluted to the final concentration in the experimental media for cell lines or diluted directly into the culture media for primary neuronal cultures. Cells were exposed to glutamate for 24 h at concentrations ranging from 50 µM to 10 mM. NMDA studies were carried out in KREBS Ringer buffer (120 mM NaCl, 5.4 mM KCl, 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 25 mM Tris HCl, 15 mM

Glucose, pH 7.4). Primary neuronal cultures were exposed to 100 μM NMDA for 5 min at room temperature and were then rinsed 3 times before a 24 h incubation (37°C) in KREBS Ringer buffer. In steroid-treated groups, steroids were present during the NMDA exposure and during every step thereafter.

Determination of Cell Viability

Cell viability was determined by trypan blue exclusion, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) conversion assay, lactate dehydrogenase (LDH) release assay, or Calcein AM/propidium iodide fluorescent staining.

Trypan blue dye-exclusion technique

The trypan blue exclusion has been described previously (Black and Berenbaum 1964; Tennant 1964). Briefly, cells were lifted using 0.02% EDTA, incubated with a equal volume of 0.4% trypan blue dye (Sigma Chemical Co.). Live cells were distinguished by exclusion of the blue dye. Each sample was counted in duplicate on a Neubauer hemacytometer within 10 minutes of dye addition.

MTS dye conversion assay

MTS and phenazine methosulfate (PMS) were obtained from Promega Corp. (Madison, WI). Immediately prior to the assay, a 333 μg/ml MTS/25 μM PMS solution was prepared, and 20 μl of this solution was added to each well of the 96 well plate. Cells were incubated at 37°C for 4 h. MTS is converted to a soluble formazan product by metabolically active mitochondria. Formation of the formazan product was measured by

the absorbance at 490 nm with a 690 nm reference wavelength. Background absorbance was determined by the absorbance of well containing media and MTS/PMS but no cells.

LDH release assay

LDH activity was determined using CytoTox 96® kit (Promega Co., Madison, WI) according to the manufacturer's instructions. LDH is a stable cytosolic enzyme that is released into the media upon cell lysis. Media were collected, centrifuged at 250 x g for 5 min to remove cell debris and was then stored at -80°C until analysis. Equal volumes of media and LDH substrate mix were incubated in a 96 well plate for 30 min at 25°C in the dark. One volume of 1 M acetic acid was added to stop the reaction. LDH activity of culture media from untreated cells was determined and used as a control. To determine 100% LDH release, or maximum toxicity, three cultures underwent three freeze/thaw cycles at -80°C and 37°C before the media was stored at -80°C for analysis.

Calcein AM/propidium iodide fluorescence assay

Calcein AM and propidium iodide were obtained from Molecular Probes (Eugene, OR). Cells were incubated with 1 μM of calcein AM and 1 μg/ml of propidium iodide in PBS (pH 7.4) for 15 minutes. Live cells were distinguished by (1) the presence of a bright green fluorescence resulting from cleavage of the calcein AM dye by intracellular esterase activity; and (2) the absence of nuclear staining by propidium iodide, which enters cells with damaged membranes and upon binding to nucleic acids produces a red fluorescence. Cells were then visualized using a flourescent Nikon® microscope with a G2-A filter. Two to three random fields were photographed, and the cells counted.

Assay Procedures

Estrogen Receptor Binding

Nuclear exchange assay. Nuclear specific ^{3}H-estradiol binding was evaluated using a modified nuclear exchange assay (Miranda et al. 1996). Cells were plated in 6-well plates for 4 h prior to experimentation. Cells were then incubated at 37°C with 2 nM ^{3}H-estradiol (Amersham, Arlington Heights, IL) in the absence and presence of 2 μM DES (Steraloids, Wilton, NH) for specific and non-specific determinations, respectively. Cells were rinsed once with ice-cold media, lysed in buffer N1 (1 mM KH_2PO_4, 3 mM $MgCl_2$, 0.25% (v/v) Triton X-100, pH 6.5) and a crude nuclei pellet isolated by centrifugation at 11,000 x g for 6 min. This pellet was rinsed once with buffer N1. The pellet was resuspended in buffer N2 (1 mM KH_2PO_4, 3 mM $MgCl_2$, pH 6.5), inverted for 5 min, and centrifuged at 11,000 x g for 6 min. Three washes with buffer N2 were done. Radioactivity was extracted in 95% ethanol, Liquiscint™ scintillation solution was added, and the mixture was counted on a Beckman LS5000 counter. Specific binding was normalized to cell number. MCF-7 cells were utilized as a positive control in all assays.

Whole cell exchange assay. Whole cell specific ^{3}H-estradiol binding was determined as previously described (Nakao et al. 1981). Cells were maintained for 24 h in phenol red free RPMI-1640 supplemented with 10% charcoal stripped FBS. Cells were then lifted, and 1 x 10^6 cells were plated per well and allowed to attach for 4 h. Cells were incubated with 2 nM ^{3}H-estradiol alone for total binding and 2 nM ^{3}H-estradiol plus 2 μM unlabeled estradiol for nonspecific binding for 1 h at 37°C. Cells were then rinsed with ice cold DPBS before lysis with 0.1 N NaOH. Liquiscint™ scintillation solution was added to the cell lysate, and the mixture was counted on a Beckman LS5000 counter. Specific binding

was normalized to cell number. MCF-7 cells were utilized as a positive control in all assays.

Cytosolic and Nuclear Protein Extractions

Cytosolic and nuclear extracts were prepared by a modified mini-extraction protocol (Schreiber et al. 1989). Cells were pelleted and resuspended in an ice cold hypotonic buffer containing 10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiotreitol (DTT), 1 μg/ml leupeptin, 1 μg/ml aprotinin and 0.5 mM PMSF (protease inhibitors from Calbiochem, San Diego, CA; all other reagents from Fisher Scientific). After incubation on ice for 15 min, NP40 was added, and the cells vortexed for 10 sec. After centrifugation at 14,000 x g for 1 min, the supernatant (cytoplasmic extract) was collected and frozen. The pellet was resupended in an extraction buffer containing 10 mM HEPES, pH 7.9, 1 mM EDTA, 10% glycerol, 1 mM DTT, 400 mM NaCl and incubated on ice for 30 min. This nuclear homogenate was then centrifuged at 14 k RPM for 15 min, and the supernatant representing the nuclear extract was frozen. Protein concentrations were determined using the Bradford method (Bradford 1976).

Electrophoretic Mobility Shift Assays

The NFκB response element double-stranded oligonucleotide (5'-AGTTGAGGGGACTTTCCCAGGC-3') and the CRE double-stranded oligonucleotide (5'-AGAGATTGCCTGACGTCAGAGAGCTAG-3'; both from Promega Corp.) corresponding to the NFκB consensus sequence and the CRE consensus sequence, respectively, were end-labled with γ-[^{32}P]ATP (Amersham) and T4 kinase (Promega

Corp.) according to the manufacturer's instructions. Labeled oligonucleotides were purified using a G25 spin column (BioRad; Hercules, CA). 8-12 μg of nuclear extract were incubated at room temperature for 20 min with 2 μg poly(dI.dC) (Amersham), 10% glycerol, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, and 15000 to 25000 cpm of ^{32}P-labeled oligonucleotides. For identification of nonspecific binding, a 100X concentration of unlabeled oligonucleotide was added 10 min prior to the addition of the labled probe. For CRE assays, 1 μl of a CREB antibody (CREB-1, Santa Cruz, Santa Cruz, CA) that does not interact with other CREB family members was added, and samples were further incubated for 30 min at room temperature. DNA-protein complexes were resolved on a 6% nondenaturing polyacrylamide gel (29:1 acrylamide to bis ratio in Tris HCL-Borate-EDTA buffer) using a Biorad Protean II electrophoresis apparatus run at 90 V for 2 h. Gels were dried and exposed to x-ray film overnight at -80°C.

Protein Assays

Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin (BSA) at concentrations ranging from 0.063 mg.ml to 1 mg/ml as a standard curve.

CHAPTER 3
17α-ESTRADIOL EXERTS NEUROPROTECTIVE EFFECTS IN VITRO

Introduction

Not only are estrogens important for the differentiation of certain brain nuclei of the brain nuclei during development (Gorski et al. 1980), but recent evidence suggests that estrogens may be important for normal brain function throughout life (Simpkins et al. 1994). In adult rats, βE2 has been shown to enhance sprouting of commissural association fibers in the hippocampal dentate gyrus after entorhinal cortex lesions (Morse et al. 1986). Estrogen environment influences the synaptology of the hippocampus because changes in synaptic density in the CA1 region are associated with endogenous (Wooley and McEwen 1992) and exogenous (Wooley et al. 1990) levels of βE2. Recently, βE2 has been shown to induce the expression of the two neurotrophic factors NGF and BDNF (Singh et al. 1995). Similarly, increases in the high-affinity uptake of choline (O'Malley et al. 1987; Singh et al. 1994), in the levels of choline acetyltransferase (Luine et al. 1975, 1980; Singh et al. 1994), and in the performance of memory-related behavioral tasks (Singh et al. 1994) have been observed after estrogen treatment of ovariectomized rats. Further, Sherwin et al. (Sherwin 1988; Phillips and Sherwin 1992) have shown estrogen enhancement of memory and cognition in women subjected to surgical menopause.

Estrogens seem to exert neurotrophic and neuroprotective effects on a variety of cell types. Toran-Allerand (1976) first reported that estrogens stimulate neurite

outgrowth of explants of the hypothalamus and preoptic area of the basal diencephalon. More recently, estrogens have been shown to protect transformed neurons and glia from the cytotoxic effects of a variety of insults, including serum deprivation (Bishop and Simpkins 1994) and hypoglycemia (Bishop et al. 1994). βE2 was particularly effective in protecting SK-N-SH neuroblastoma cells from the neurotoxic effects of serum deprivation (Bishop and Simpkins 1994).

The studies cited above have used the β-isomer of E2, which is known to interact potently with the ER (Korenman 1969; Lubahn et al. 1985), and the ER-βE2 complex binds with a long duration to the ERE (Clark et al. 1982). By contrast, αE2 binds weakly to the ER, and the αE2-ER complex only transiently binds to the ERE (Korenman 1969; Clark et al. 1982; Lubahn et al. 1985). When administered acutely, αE2 has only weak (Korenman 1969) or no (Huggins et al. 1954; Kneifel et al. 1982) activity in peripheral estrogen-responsive tissues but seems to exert uterotropic effects with administration chronically at high doses (Clark et al. 1982; Clark and Markaverich 1983).

The present study was undertaken to compare the relative activities of the β- and α-isomers of estradiol to examine whether the neuroprotective effect of estrogens was mediated by the classical ER-mediated pathway. We examined the neuroprotective effects of both estradiol isomers in both the SK-N-SH neuroblastoma cell line and rat primary cortical neurons. Further, multiple toxicities (serum deprivation, Aβ exposure, and EAA) were evaluated to determine if the neuroprotective effects of estrogens were insult-specific. Finally, other steroids were tested to determine the specificity of this steroid-mediated neuroprotection for estrogens.

Materials and Methods

Experimental Media

Experiments were initiated by the back-culturing of SK-N-SH cells. Cells were suspended in the appropriate treatment medium and plated at either 2.5 x 10^5 cells/well (low density plating) or 1 x 10^6 cells/well (high density plating) in 24 well Falcon plates (Fisher Scientific, Orlando, FL). In all serum-deprivation studies, cells were cultured in RPMI-1640 media (SF group), RPMI-1640 media supplemented with 10% FBS (FBS group), or RPMI-1640 media supplemented with one of the following steroids at the dose(s) indicated: βE2 (0.2 or 2 nM; Pharmos, Alachua, FL); αE2 (0.2 or 2 nM; Sigma, St. Louis, MO); cholesterol (CHOL, 2 nM; Steraloids, Wilton, NH); testosterone (TEST, 2 nM; Steraloids); dihydrotestosterone (DHT, 2 nM; Steraloids); or corticosterone (CORT, 2 nM; Steraloids). In one study, a 10-fold molar excess of tamoxifen (20 nM; Steraloids) was administered at the same time as βE2 (2 nM) or αE2 (2 nM). In another study, tamoxifen (0 to 200 nM) was administered in the presence or absence of βE2 (2 nM). In all Aβ studies on SK-N-SH cells, cells were cultured in RPMI-1640 with 10% FBS (control group) or RPMI-1640 supplemented with 10% FBS and the indicated doses of Aβ (25-35), 2 nM βE2 and/or 2 nM αE2. Exposure to toxins is described in Chapter 2. All steroids were dissolved initially at 1 mg/ml in absolute ethanol and diluted to the indicated final concentration in the experimental media. To control for possible ethanol effects in the steroid-treated wells, all non-steroid treated groups were supplemented with absolute ethanol at a concentration of 0.0001% - 0.01% (v/v) to correlate with the highest dose of steroid used. The experiments depicted in Figure 3-8 B and D utilized either hydoxypropyl-β-cyclodextrin (HPCD)-encapsulated βE2 (Sigma Chemical Co.) or HPCD-

encapsulated αE2 (supplied by Dr. Laszlo Prokai, University of Florida) at a final steroid concentration of 133 nM. The vehicle control used in these studies was 2.78 μM HPCD.

Quantitation of Cell Viability

Cell viability on SK-N-SH cells was assessed at 24, 48, and/or 96 hr of treatment using the trypan blue dye exclusion method as described in Chapter 2. Cell viability on the primary cortical neurons was assessed by either a LDH release assay or the calcein AM/propidium iodide assay as described in Chapter 2.

Statistical Analysis

All statistical analyses were performed on raw data. Each well or dish constituted an independent sample. The significance of differences among groups was determined by one-way ANOVA. Planned comparisons between groups was done by Scheffe's F-test. For all tests, $p < 0.05$ was considered significant.

Results

Serum Deprivation Toxicity

Serum deprivation had a marked effect on the viability of SK-N-SH cells at both high and low plating density (Table 3-1). At high plating density (1×10^6 cells/well) and in the presence of FBS, 52-60% of cells died in the first 24 hr, and then live cell number increased slightly for the next 24 hr. By contrast, serum deprivation at high plating density resulted in a loss of 77-86% plated cells by 24 hr and 83-91% of cells by 48 hr. At low plating density (0.25×10^6 cells/well), the presence of FBS maintained live cell number constant through 48 hr of the studies. Serum deprivation at low plating density reduced

Table 3-1. Effects of Serum Deprivation and 17α-Estradiol on SK-N-SH Cell Number after Plating at High and Low Density.

Treatment	Plating density	Culture duration (hr) 24	48
		Cells/well ($\times 10^3$)	
FBS	1000	405 ± 21	452 ± 51
SF	1000	140 ± 11*	87 ± 7*
FBS	1000	480 ± 50	530 ± 100
SF	1000	230 ± 20*	130 ± 20*
αE2 (2 nM)	1000	480 ± 50	300 ± 60
FBS	250	235 ± 12	232 ± 6
SF	250	42 ± 2*	26 ± 2*

Cells were plated at the indicated density and either maintained in RPMI-1640 with 10% FBS (FBS group) or serum-free (SF) RPMI-1640 (SF and αE2 groups). Cell number was determined 24 or 48 h later. *$p < 0.05$ versus FBS group. Given are mean ± sem for 6 wells per group. One experiment included in this table was designed and executed by Jean Bishop.

live cells by 82% at 24 hr and by 91% at 48 hr. The effects of FBS and serum deprivation on SK-N-SH cells at a high plating density are similar to previously reported observation using flasks, as opposed to wells, for culturing of this line of neuroblastoma cells (Bishop and Simpkins 1994).

Previously a potent cytoprotective effect of βE2 was observed on SK-N-SH cells cultured at high density in SF media (Bishop and Simpkins 1994). αE2, the optical isomer of βE2, was tested in an initial study as a control for the stereoselective, receptor-mediated effects of estradiol (Table 3-1). As reported previously for βE2, αE2 partially or completely prevented the death of SK-N-SH cells in response to serum deprivation. The presence of 2 nM αE2 increased live cell number about 2-fold in cultures grown for

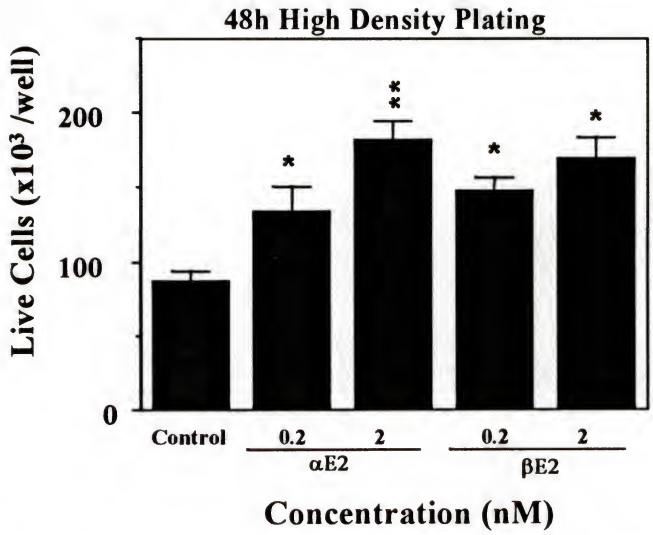


Figure 3-1. Effects of α- and β-estradiol on live SK-N-SH cell number after plating of cells at high density. Cells were plated at 1×10^6 cells per well, and 48 h later cell number was determined. All wells were deprived of serum for the entire incubation period. All wells were treated either with αE2 or βE2 at the concentrations indicated or with no steroid (Control). The FBS group in this study contained 452 ± 51 ($x10^3$ cells/well). *$p < 0.05$ versus control; **$p < 0.05$ versus control and αE2 groups at 0.2 nM. There were no differences between αE2 and βE2 groups at the same doses of the steroid. Depicted are mean ± sem for 4 wells per group.

24 and 48 hr as compared to the SF group, suggesting a neuroprotective effect of the α-isomer.

In a subsequent study, the effectiveness of αE2 effect was compared to that of the naturally occurring βE2 (Figure 3-1). With initial plating at high density (1×10^6 cells/well), both the α- and β-isomers caused a dose-dependent increase in live cell number at 48 hr in culture (Figure 3-1). For both isomers, serum-deprived SK-N-SH cell number was increased by 65% at the 0.2 nM dose and by 88% at the 2 nM dose compared to the nonsteroid-treated cells.

The time course of the effect of αE2 and βE2 was evaluated in serum-deprived SK-N-SH cultures plated at a low density (Figure 3-2). At 24 hr of treatment (Figure 3-2A), the low concentrations of both E2 isomers were ineffective in preventing the serum

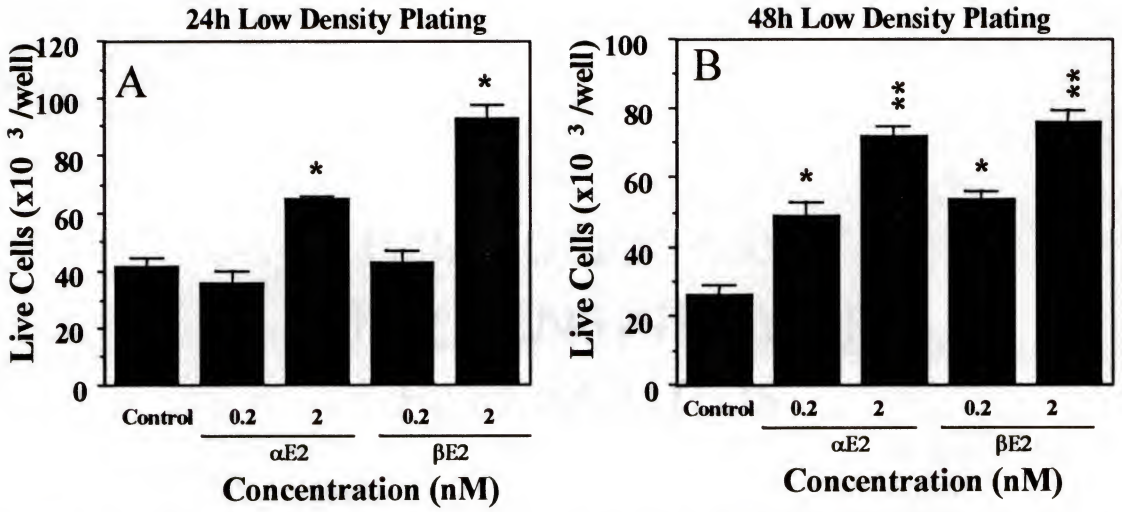


Figure 3-2. Effects of α- and β-estradiol on live SK-N-SH cell number after plating of cells at low density. Cells were plated at 0.25×10^6 cells per well, and cell number was determined at 24 (A) or 48 hr (B) later. All wells were deprived of serum for the entire incubation period. Wells were treated either with no steroid (Control) or with αE2 or βE2 at the concentrations indicated. The FBS groups in this study contained (A) 235 ± 12 and (B) 232 ± 6 ($x10^3$ cells/well).*$p < 0.05$ versus control; **$p < 0.05$ versus control and the respective isomer at the 0.2 nM concentration. Depicted are mean ± sem for 6 wells per group for both studies.

deprivation-induced cell loss. However, the 2 nM concentration produced a 54% increase in live cell number for the α-isomer and a 116% increase for the β-isomers. At 48 hr of culture, both α- and β-isomers caused a 86-106% and 172-189% increase in number of trypan blue-excluding cells at the 0.2 and the 2 nM concentrations, respectively (Figure 3-2B).

Tamoxifen alone had no effect on live cell number in serum-deprived cultures when administered at concentrations ranging from 2 to 200 nM (Figure 3-3A). Coadministration of a 10-fold molar excess of tamoxifen with βE2 or αE2 reduced the neuroprotective effect of the estrogen by 39 and 32%, respectively (Figure 3-3C). The tamoxifen effect on βE2 neuroprotection was not evident at 2 nM, was 32% at 20 nM, and was not enhanced further at 200 nM (Figure 3-3B).

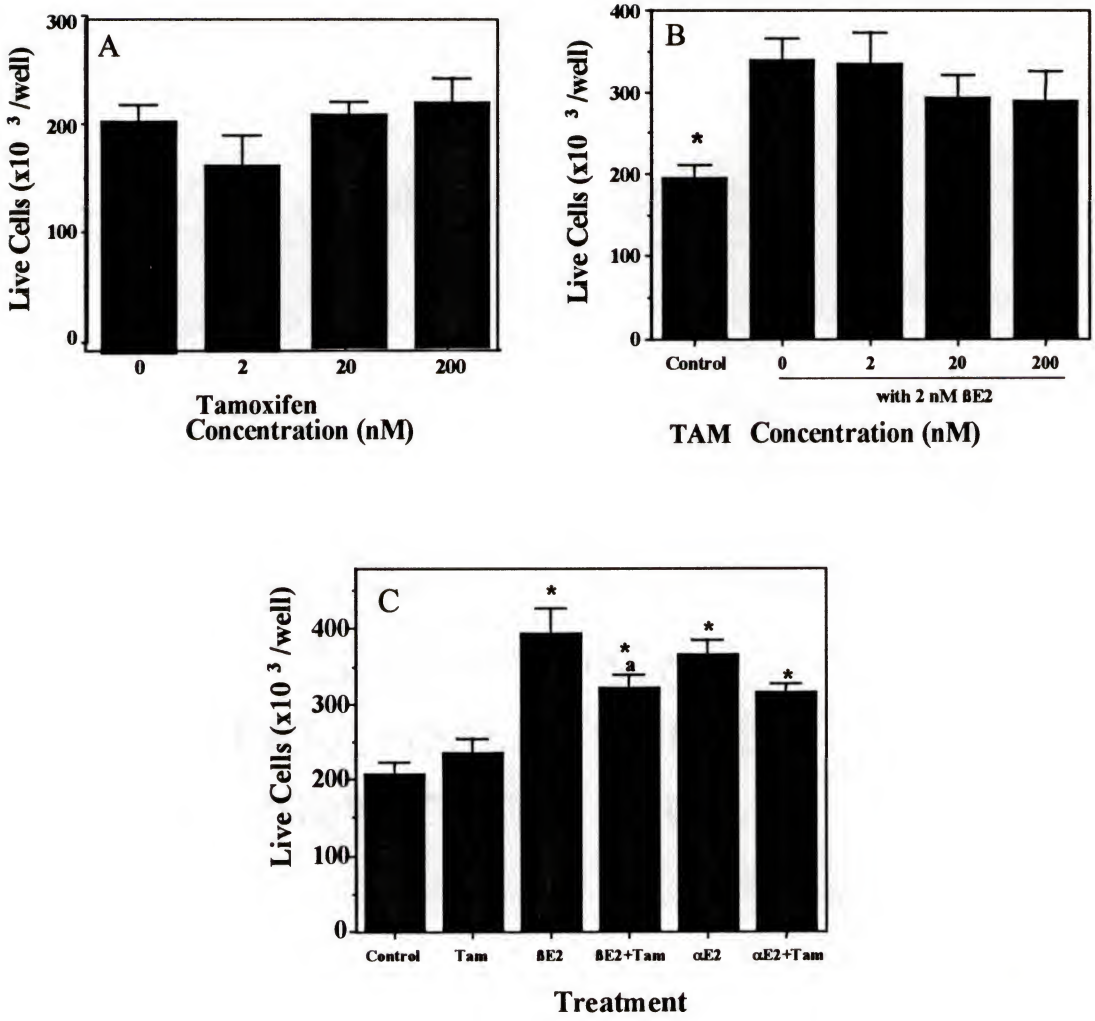


Figure 3-3. Effects of treatment with tamoxifen (TAM, 0-200 nM in A and B and 20 nM in C), β-estradiol (βE2, 2 nM), α-estradiol (αE2, 2 nM), or their combination on live cell number. SK-N-SH cells were plated at 1×10^6 cell per well, and cell number was determined 48 hr later. Cells were deprived of serum during the entire incubation period. In (A), no differences were statistically significant. In (B), *$p < 0.05$ versus all other groups. No other differences were significant. In (C), *$p < 0.05$ versus Control (SF) and TAM groups. a = $p < 0.05$ versus the βE2 group. In these studies, the FBS groups averaged 656 ± 43 ($x10^3$ cells/well). Depicted are mean ± sem for 4-5 wells per group.

The specificity of this estrogen effect on serum-deprived SK-N-SH cells was assessed in both high and low density cultures by evaluating a variety of steroids, including cholesterol, progesterone, testosterone, dihydrotestosterone, and corticosterone. At 48 h of culture, the time of the peak effect of both α- and βE2, none of the substances tested protected cells from the cytotoxic effects of serum deprivation (Table 3- 2).

Table 3-2. Effects of Various Steroids and Plating Density on Live SK-N-SH Cell Number at 48 h.

	Low plating density	High plating density
Treatment	Live cell number ($\times 10^3$)	
Serum-free control	22.4 ± 1.4	94.4 ± 7.3
Cholesterol	18.3 ± 1.1	64.8 ± 3.9
Progesterone	21.3 ± 0.7	57.6 ± 6.2*
Testosterone	20.0 ± 4.2	87.2 ± 6.3
Dihydrotestosterone	20.7 ± 2.6	90.0 ± 7.7
Corticosterone	19.7 ± 1.3	68.8 ± 6.9

All cultures were deprived of serum and exposed to the respective steroid (2 nM) during the entire 48 h incubation period. Low and high plating density are 2.5×10^5 and 10^6 cells/well. *$p < 0.05$ versus control group; Given are mean ± sem for n=5-8 wells per group.

β-Amyloid Toxicity

We further assessed the ability of estrogens to protect both rat primary cortical neurons and SK-N-SH cells from the toxic effects of Aβ exposure. Four days of exposure to Aβ (25-35) resulted in a dose-dependent death of SK-N-SH cells with a LD_{50} of 28.9 μM (Figure 3-4). Rat primary cortical neurons were more sensitive to Aβ (25-35)-induced toxicity with a 48 h exposure to a 5 μM dose resulting in 71% reduction in live cell number (Figure 3-5). This neurotoxic fragment of Aβ caused cell shrinkage, a loss of

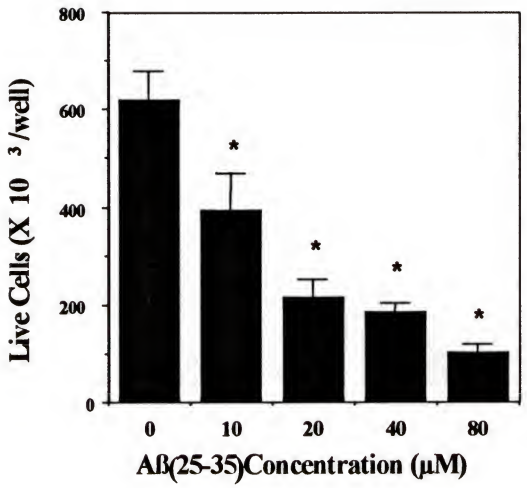


Figure 3-4. Effects of dose of Aβ (25-35) on live SK-N-SH cell number. Cells were plated at 10^6 cells/ml and were exposed to Aβ (25-35; Lot ZM500) at the dose indicated. Live cell number was determined 4 d later. *p<0.05 versus the 0 (control) group. Depicted are mean ± sem for 4 wells per group. Dr. Christopher de Fiebre and Nancy de Fiebre aided in the calculation of the LD_{50} concentration from this data. This experiment was performed in collaboration with K.E. Gridley.

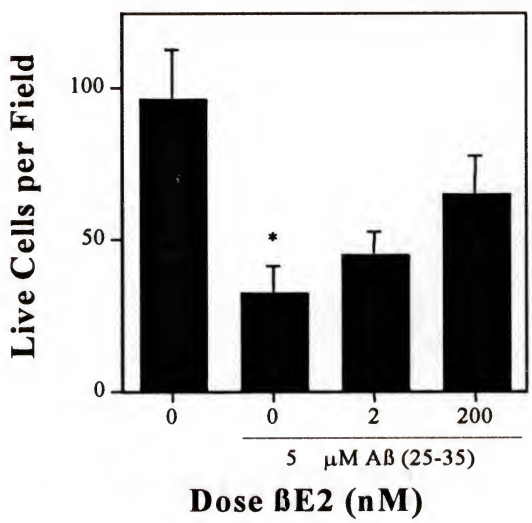


Figure 3-5. Effects of β-estradiol on Aβ (25-35; 5 µM; lot QM501) toxicity in rat primary cortical neurons. Experiments were initiated when neurons were 10 d in culture and neurons were exposed to the steroid and Aβ for 48 h. *p<0.05 versus control (no Aβ) group. No other differences were significant at the 0.05 level. Depicted are mean ± sem for 4 dishes per group. Dr. Edwin Meyer aided in the calculating the molarity of Aβ used in this experiment.

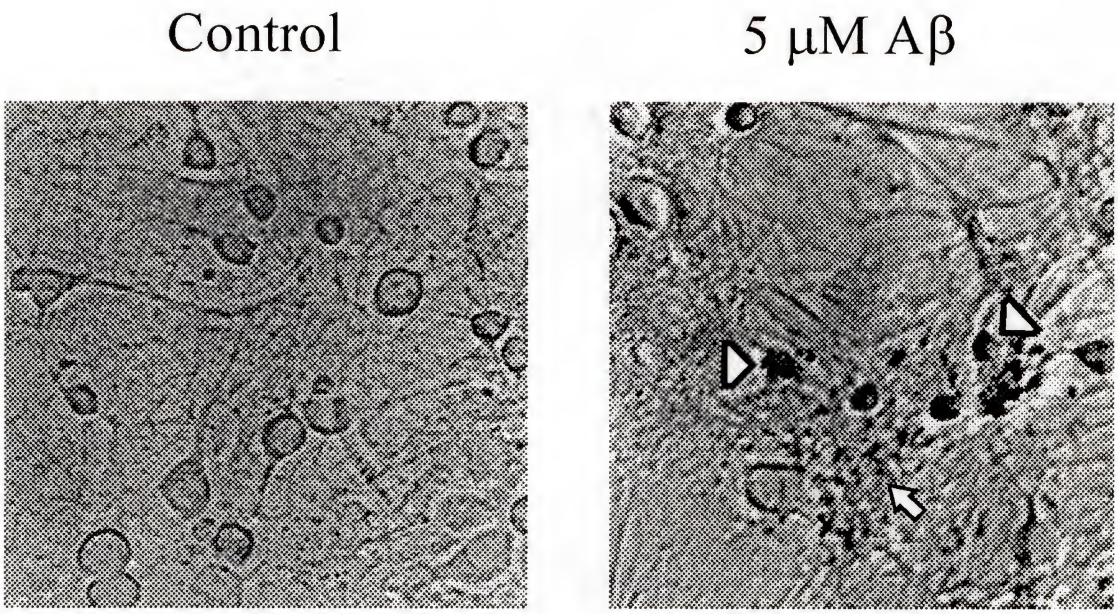


Figure 3-6. Photomicrographs of representative primary rat cortical neurons exposed to 5 μM Aβ (25-35) for 48 h. Experiments were initiated when neurons were 10 d in culture. Cells were photographed at 200x total magnification. Note the phase-dark, shrunken neurons (triangle) and the accumulation of debris (arrow) in the Aβ dish.

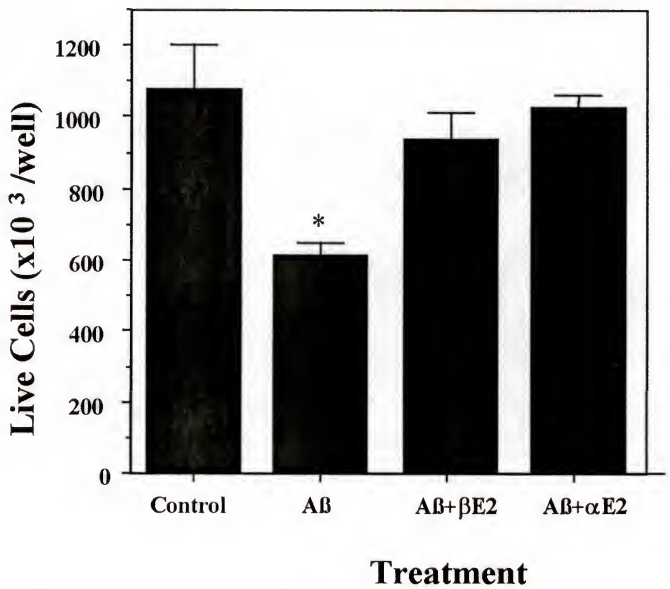


Figure 3-7. Effects of β-estradiol (2 nM) and 17α-estradiol (2 nM) on the Aβ (25-35; 20 μM; lot QM501) toxicity. SK-N-SH cells were plated at 10^6 cells/well and were exposed to vehicle, βE2, or αE2. Live cell number was determined 4 d later. *$p<0.05$ versus control group, βE2 group, and αE2 group. Depicted are mean ± sem for 5 wells per group. K.E. Gridley assisted in counting this experiment.

Table 3-3. Effects of β-Estradiol and α-Estradiol on SK-N-SH Cell Number.

Treatment	Live cell number ($x10^3$/well)
Control	1082 ± 117
βE2 (2 nM)	1034 ± 116
αE2 (2 nM)	1126 ± 75

Cells were plated at 10^6 cells/well and were exposed to vehicle, βE2, or αE2. Live cell number was determined 4 d later. No differences are statistically significant. Depicted are mean ± sem for 5 wells per group.

phase-bright neurons, fragmentation of the neurites, and the accumulation of cellular debris in the culture wells (Figure 3-6).

βE2 conferred a dose-dependent protection on these primary neurons with a 2 nM dose protecting 17% and a 200 nM dose protecting 43% of the neurons (Figure 3-5). The βE2- treated primary cultures showed evidence of more neuronal-type cell morphology and intact neurites (data not shown). The addition of 2 nM βE2 protected an average of 68% of SK-N-SH cells from the toxicity induced by three different lots (ZM500, ZM501, QM501, respectively) of Aβ (25-35; Figure 3-7 and data not shown). A 2 nM dose of αE2 was as efficacious as 2 nM βE2 in attenuating the cell loss due to Aβ (25-35) treatment (Figure 3-7). Neither βE2 nor αE2 exerted a mitogenic effect on SK-N-SH neuroblastoma cells with a 4 d treatment in the absence of Aβ (25-35; Table 3-3).

Excitotoxicity

Both βE2 and αE2 were evaluated for neuroprotective efficacy against glutamate, NMDA, and AMPA toxicity in rat primary cortical neurons (Figure 3-8). The effects of

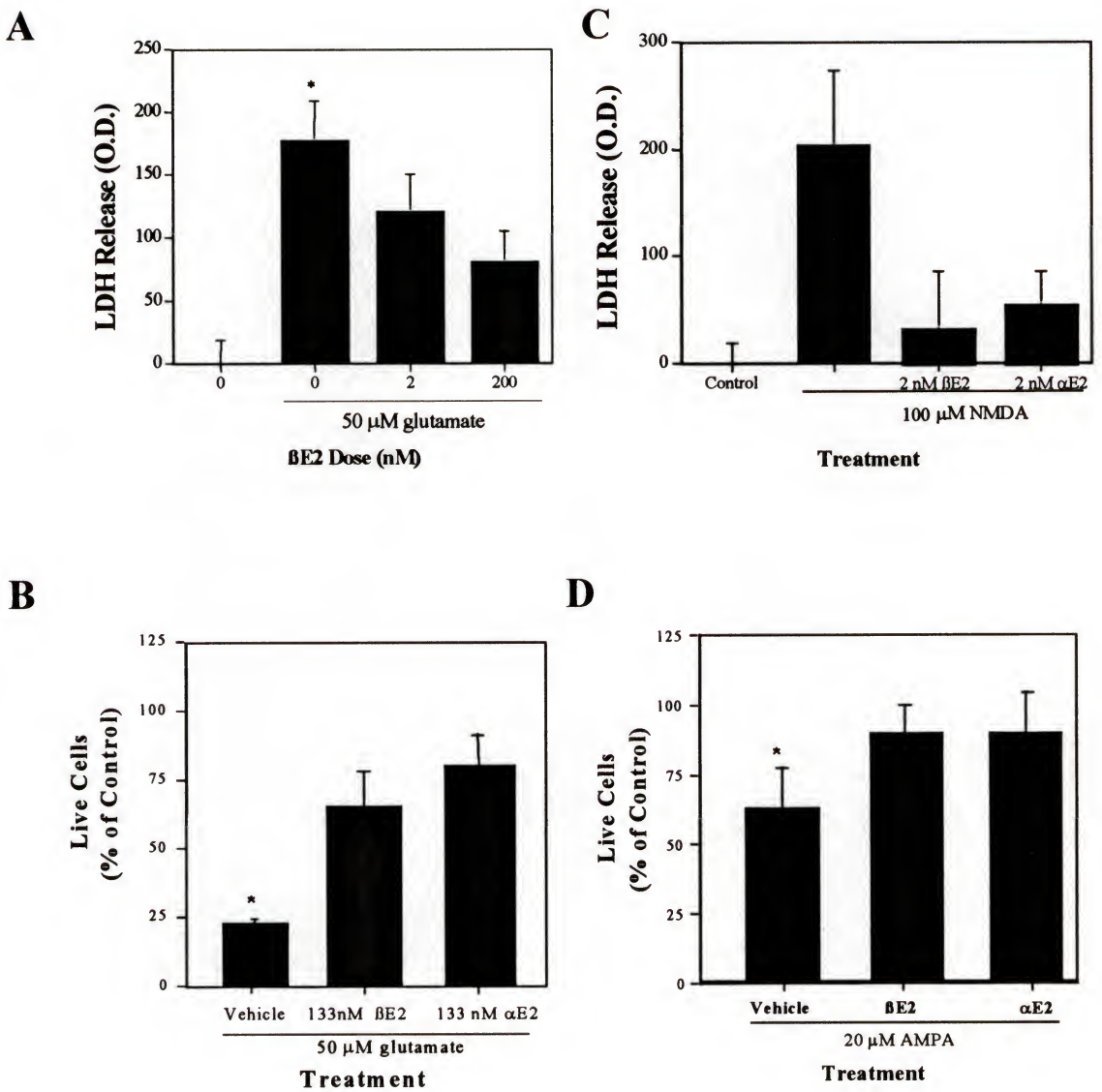


Figure 3-8. Effects of both β-estradiol and α-estradiol on survival of rat primary cortical neuron from excitotoxic insults. Experiments were initiated when neurons were 10-12 d in culture and neurons were exposed to the steroid concurrent with the toxin (A and C) or 24 h prior to the toxin (B and D). In both (A) and (B), neurons were exposed to 50 μM glutamate for 24 h and viability was assessed at the end of the glutamate treatment. In (C), neurons were exposed to 100 μM NMDA for 5 min and viability determined 24 h later. In (D), neurons were exposed to 20 μM AMPA for 24 h and viability was determined at the end of the AMPA treatment. *$p<0.05$ versus no toxin control group (100% of Control value in B and D). Depicted are mean ± sem for 11-16 wells per group (A and C) or 5-6 wells per group (B and D). (B) and (D) are presented as percent of control cell number per field (control = 100%). The studies in (B) and (D) were performed by Larissa L. Zaulynov. My role in these experiments included assisting in experimental design, assay development, aid in calculations to achieve correct doses, camera assistance and data analysis and interpretation. All statistical analysis was performed on raw data.

estrogens on glutamate toxicity were determined using two different viability assays. The dose of glutamate used (50 μM) resulted in 32% cell death as determined by a media LDH assay and 76% reduction in live cell number as determined by calcein AM/propidium iodide fluorescence (Figure 3-8 A and B). A 2 nM and 200 nM dose of βE2 resulted in a 32% and 56% reduction in media LDH activity from cultures treated with glutamate alone, respectively (Figure 3-8A). Similarly, when viability was determined using Calcein AM/propidium iodide fluorescence, pretreatment of neurons with βE2 (133 nM) and αE2 (133 nM) protected 58% and 82% of cells, respectively (Figure 3-8B). Exposure of neurons to 20 μM AMPA resulted in 34% decrease in live cell number, and pretreatment with 133 nM of βE2 and αE2 protected 85% and 86% of these neurons, respectively (Figure 3-8D). A 5 min exposure to 100 μM NMDA drastically increased media LDH activity in a manner that was reduced by 84% and 72% with concurrent treatment with 2 nM βE2 and αE2, respectively (Figure 3-8C).

Discussion

The present study demonstrates for the first time that αE2, a weak ER agonist (Huggins et al. 1954; Korenman 1969; Clark et al. 1982; Kneifel et al. 1982; Clark and Markaverich 1983; Lubahn et al. 1985), is as effective as βE2 in attenuating neuronal death due to serum deprivation, Aβ toxicity, and excitotoxic insults. These results suggest that the estrogenic effect on neuronal survival is mediated via a mechanism that does not require binding to the nuclear ER. This conclusion is consistent with the observation that the simultaneous addition of a 10-fold molar excess of the ER antagonist, tamoxifen, reduced the neuroprotective effects of both estrogens by only one-third, and a 100-fold

molar excess of tamoxifen had no additional effect on the βE2 response. The protective effect is not attributable to a general steroid structure, because cholesterol, a progestin, androgens, and a glucocorticoid were ineffective in protecting SK-N-SH cells from the effects of serum deprivation. Concentrations of progesterone (PROG) and corticosterone (CORT) as high as 200 nM were ineffective in protecting SK-N-SH cells from the cytotoxic effects of serum deprivation (Figure 4-1).

Estrogens could attenuate excitotoxicity by direct interaction with either NMDA or AMPA/kainate receptors. βE2 has been shown to have direct effects on both AMPA/kainate currents (Wong and Moss 1992) and NMDA currents (Weaver et al. 1997). Further, the neurotrophic effects of estrogens are attenuated by NMDA receptor antagonists (Wooley and McEwen 1992; Murphy and Segal 1996; Briton et al. 1997a). A direct attenuation of excitotoxicity may result in protection from other insults, as vulnerability to excitotoxicity is increased with both serum-deprivation (Atabay et al. 1996) and Aβ exposure (Koh et al. 1990).

Our data suggest that the neuroprotective effects of estrogens apply to both Aβ toxicity, which has been implicated in AD (for review see Selkoe 1994), and excitotoxicity, which may be involved in several disease states, including stroke and AD (for review see Lipton and Rosenberg 1994). We demonstrate that both βE2 and αE2 attenuate neuronal death induced by serum deprivation, Aβ toxicity, and glutamate toxicity mediated by either NMDA or AMPA receptor activation. This would suggest that estrogens either protect neurons through multiple mechanisms of action or that estrogens affect a pathway common to all three insults.

In these studies, estrogens exerted neuroprotective effects at physiologically relevant concentrations. Both βE2 and αE2 protected neuronal cells from the toxic effects of serum deprivation at concentrations as low as 0.2 nM. This concentration corresponds to the average circulating βE2 levels during the menstrual cycle (Baird and Guevara 1969; Mishell et al. 1971). Doses of both steroids as low as 2 nM attenuated toxicity induced by Aβ and EAAs. This concentration of total circulating estrogens may be seen in women during ovulation, pregnancy, and post-menopausal estrogen replacement therapy (Baird and Guevara 1969; Mishell et al. 1971; Whittaker et al. 1980; Castelo-Branco et al. 1995). Studies utilizing injection of radiolabled βE2 indicate that 40 to 50 % of total plasma βE2 is rapidly extracted by the brain in the presence of physiological levels of sex hormone binding globulin (SHBG; Pardridge et al. 1980; Gambone et al. 1982; Sakiyama et al. 1982; Laufer et al. 1983). Further, brain concentrations of βE2 may exceed plasma levels (Rahimy et al. 1990), and in the brain, βE2 is concentrated in neurons including those of the cortex and hippocampus (Toran-Allerand et al. 1992). These data indicate that E2 concentrations observed in women can exert a neuroprotective effect.

The doses at which we demonstrate neuroprotection are in the range of those reported for neurotrophic effects of βE2 (Toran-Allerand 1976 1980; Faivre-Bauman et al. 1981; Nishizuka and Arai 1981; Toran-Allerand et al. 1983; Morse et al. 1986; Wooley et al. 1990; Wooley and McEwen 1992; Briton 1993). βE2 meditated neuroprotection with low nM doses of the steroid against serum deprivation, hypoglycemia, and glutamate toxicity has been previously reported (Bishop and Simpkins 1994; Bishop et al. 1994; Singer et al. 1996). Both Behl et al. (1995) and Goodman et al. (1996) have also

demonstrated neuroprotective effects of βE2 against glutamate toxicity but required μM doses of the steroid. However, to our knowledge, this is the first observation that αE2 exerts neuroprotective effects.

The observed neuroprotective effects of estrogens are not assay dependent, as we demonstrate estradiol-mediated neuroprotection using multiple viability assays. Both the trypan blue dye exclusion assay and the calcein AM/propidium iodide staining technique quantitate live cell number based upon membrane integrity and cell size/shape (Black and Berenbaum 1964; Tennant 1964; Haughland 1996). The LDH release assay is a measure of cell death determined by the release of a functional cytosolic enzyme into the culture media (Koh and Choi 1987). Neuroprotective effects of βE2 can also be detected using mitochondrial activity as a marker for cell viability (Figure 5-4).

Of these assays, LDH is a less sensitive measure of apoptotic cell death, as membrane integrity remains intact in apoptotic cells (Kerr et al. 1972). Both the trypan blue dye exclusion assay, and the calcein AM/propidium iodide assay, show reductions in live cell number from both apoptotic and necrotic cell death. Although these two assays stain dead cells due to lack of membrane integrity, morphological criteria (approximate cell size) are included in the assessment of live cell number and prevent apoptotic bodies from contributing to live cell number. In this study, cell death due to glutamate toxicity was significantly larger as determined by the calcein AM/propidium iodide assay than as determined by the LDH release assay. Interestingly, βE2 conferred roughly equivalent protection of the cells in both assays, suggesting that βE2 is attenuating both the necrotic and apoptotic components of glutamate-induced neuronal death.

At low and high density plating, both βE2 and αE2 protected SK-N-SH cells from the toxic effects of serum deprivation. The comparatively lower protective effects of the two isomers in the high density cultures is likely attributable to either (1) the fourfold lower steroid-to-cell ratio in the high density cultures or (2) the plating of cultures at near confluence in the high plating density cultures, a condition that results in the loss of cells because of competition for surface area of the plates. Nonetheless, the data indicate that at both high and low plating density, both estradiol isomers are neuroprotective against serum deprivation.

I considered the possibility that the observed effects of αE2 may have been subsequent to its conversion by 17α-oxidoreductase to estrone and subsequent reduction of the 17-ketone to βE2 (Breuer and Schott 1966; Williams and Layne 1967). However, this is an unlikely because 17α-oxidoreductase activity is low in human tissue (Breuer and Schott 1966; Williams and Layne 1967) relative to that seen in other species (Mulay et al. 1968; Ivie et al. 1986). The conversion of αE2 to estrone is reported to be <6%, both *in vivo* and in liver homogenates *in vitro* (Breuer and Schott 1966; Williams and Layne 1967). In our *in vitro* system, such a low conversion of αE2 to estrone would express itself as a markedly lower potency for the α-isomer. Our observation of equal effectiveness of the two isomers indicates that metabolic activation of αE2 is not necessarily involved in its cytoprotective effects, though we cannot rule out multiple active agents with similar potencies.

Nongenomic actions of βE2 are now well described. Direct effects of estrogens on neuronal membranes have been hypothesized to involve specific membrane receptors (Pietras and Szego 1979; Razandi et al. 1999); βE2 has been shown to increase

hippocampal slice CA1 field potentials (Teyler et al. 1980) and to potentiate excitatory post-synaptic potentials of CA1 neurons within 2 min of its addition to slices (Wong and Moss 1991, 1992), a time too short to involve a transcriptional mechanism. Local application of βE2 also has been shown to alter Ca^{2+} fluxes in granulosa cells (Morley et al. 1992) and endometrial cells (Nemere and Norman 1992) and to increase Ca^{2+} currents in GH3 anterior pituitary cells (Richie 1993). As such, we presume that both βE2 and αE2 can exert their neuroprotective effects via a mechanism that does not require an interaction of the steroid with the estrogen receptor and the subsequent activation of genes. Indeed, recent evidence for the antioxidant activity of estradiol in a cell line that lacks the ER (Behl et al. 1995) supports this contention.

The potential relevance of the observed neuroprotective effects of estrogen is demonstrated by the epidemiologically observations that post-menopausal ERT correlates with a decreased incidence of AD (Paganini-Hill and Henderson 1994; Tang et al. 1996) and three reports of improvements in symptoms of AD in some women with estrogen replacement therapy (Fillit et al. 1986; Honjo et al. 1989; Ohkura et al. 1994). The observed neuroprotective effects of the αE2 isomer suggest that this compound may be particularly useful in achieving a selective neuroprotective action of estrogens without overstimulation of peripheral estrogen-responsive tissues.

CHAPTER 4
EFFECTS OF CORTICOSTERONE AND PROGESTERONE ON THE NEUROPROTECTIVE EFFECTS OF ESTRADIOL

Introduction

Modulation of neuronal survival and vulnerability by both adrenal and gonadal steroids is increasingly being described. Estrogens have been shown to attenuate the neurotoxicity associated with exposure of cultured neurons to Aβ (Behl et al. 1995; Goodman et al. 1996; Green et al. 1996; Chapter 3), EAAs (Goodman et al. 1996; Singer et al. 1996; Zaulynov et al. 1999; Chapter 3), serum-deprived conditions (Bishop and Simpkins 1994; Green et al. 1997a) and a variety of other inducers of oxidative damage (Behl et al. 1995; Goodman et al. 1996; Behl et al. 1997b). In animal models of toxicity, βE2 similarly exerts neuroprotective effects (Rabbani et al. 1997; Shi et al. 1997; Simpkins et al. 1997; Dubal et al. 1998; Zhang et al. 1998). In addition, ERT in post-menopausal women reduces the risk of AD (Paganini-Hill and Henderson 1994; Tang et al. 1996; Kawas et al. 1997) and has been reported to improve cognitive function in women diagnosed with AD in several small clinical trials (Fillit et al. 1986; Honjo et al. 1989; Ohkura et al. 1994a; Ohkura et al. 1994b).

Several studies indicate that glucocorticoids increase the vulnerability of neurons, specifically hippocampal neurons, to excitotoxic death, oxidative damage, and age-related neuronal degeneration (for review see Landfield et al. 1994; Sapolaky 1994). Several studies indicate a dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis during AD resulting in elevations of circulating corticosteroids (Hatzinger et al. 1995).

The effects of progestins on neuronal survival are less well delineated. A neuroprotective effect of PROG administration has been described in animal models of traumatic brain injury and acute ischemia (Roof et al. 1993; Jiang et al. 1996; Roof et al. 1997; Gonzalez-Vidal et al. 1998) that has been attributed to a reduction in the injury-induced edema (Roof et al. 1993; Duvdevani et al. 1995; Roof et al. 1996). In neuronal cultures, the effects of PROG on neuronal survival are mixed, with PROG attenuating excitotoxicity in primary hippocampal cultures (Goodman et al. 1996), but not affecting the survival of either primary hippocampal neurons (Goodman et al. 1996) or a hippocampal cell line (Behl et al. 1995) in the face of oxidative stress.

Several neuronal effects of estrogens and progestins are known to interact (for review see McEwen et al. 1990). For example, βE2 increases hippocampal dendritic spine density in ovariectomized rats, whereas PROG-treatment of βE2-primed rats, initially accentuates the βE2-induced increase before resulting in an overall decrease in dendritic spine density (Woolley and McEwen 1993). Estrogens and glucocorticoids can have opposing effects on neuronal susceptibility to toxins. The purposes of this study were to determine the effects of PROG and CORT treatment on the survival of serum-deprived SK-N-SH cells and, further, to determine if either of these steroids alter the degree of neuroprotection conferred by estrogens in this model.

Materials and Methods

Experimental Media

Experiments were initiated by the back-culturing of SK-N-SH cells. Cells were suspended in the appropriate treatment medium, and cells were plated at 1 x 10^6 cells/well

in 24 well Falcon plates (Fisher Scientific). In all serum-deprivation studies, cells were cultured in RPMI-1640 media (Control group), RPMI-1640 media supplemented with 10% FBS (as a positive control), or RPMI-1640 media supplemented with a combination of the following steroids at the dose(s) indicated: βE2 (0.2 or 2 nM; Pharmos, Alachua, FL); αE2 (0.2 or 2 nM; Sigma); CORT (0.2 - 2 nM; Steraloids); or PROG (0.2-2 nM; Steraloids). All steroids were dissolved initially at 1 mg/ml in absolute ethanol and diluted to the indicated final concentration in the experimental media. To control for possible ethanol effects in the steroid-treated wells, all non-steroid treated groups were supplemented with absolute ethanol at a concentration of 0.0001% - 0.01% (v/v) to correlate with the highest dose of steroid used.

Quantitation of Cell Viability

Cell viability on SK-N-SH cells was assessed at 48 h of treatment using the trypan blue dye exclusion method as described in Chapter 2.

Statistical Analysis

The significance of differences among groups was determined by one-way ANOVA. Planned comparisons between groups were done by Scheffe's F-test. For all tests, $p < 0.05$ was considered significant.

Results

Neither PROG nor CORT at concentrations ranging from 0.2 to 200 nM increased the survival of SK-N-SH cells under conditions of serum deprivation (Figure 4-1). At 2 and 20 nM doses, PROG caused a slight potentiation of the serum deprivation-induced

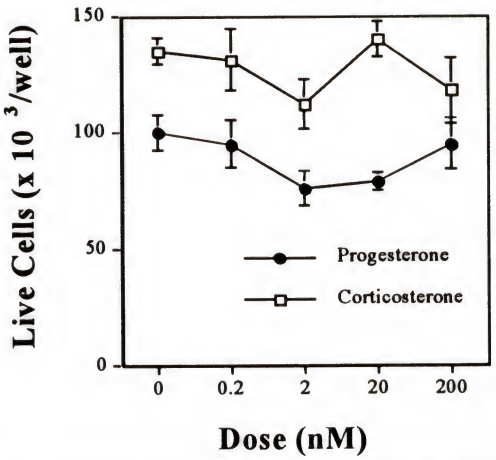


Figure 4-1. Effects of progesterone and corticosterone on live SK-N-SH cell number under conditions of serum deprivation. Cells were plated at 1 x 10^6 cells per well and 48 h later cell number was determined. All wells were deprived of serum for the entire incubation period. Wells were treated with the indicated doses of PROG or CORT concurrent with serum deprivation. No steroid doses were significantly different from the 0 nM groups. Depicted are mean ± sem for 6 wells per group.

toxicity that was not statistical significant but was seen in 6 different studies at both high (1 x 10^6 cells/well) and low (0.25 x 10^6 cells/well) plating densities (Figures 4-1, 4-2, Table 3-2). Similarly, the 2 nM dose of CORT showed a slight but not statistically significant decrease in serum-deprived SK-N-SH viability that was replicated in 5 different studies at both high and low plating densities (Figures 4-1 and 4-3, Table 3-2).

As previously reported (Chapter 3), both βE2 and αE2 caused a concentration-dependent increase in survival of serum-deprived SK-N-SH cells with a 2 nM concentration of each steroid increasing live cell number 127% and 121%, respectively (Figures 4-2, 4-3). PROG (2 nM) did not alter the neuroprotective effects of either βE2 or αE2 (Figure 4-2). The effect of PROG on estradiol neuroprotection was also evaluated under low plating density. A 2 nM concentration of βE2 caused a 251% and a 235% increase in live cell number following serum-deprived in the presence and absence of 2 nM

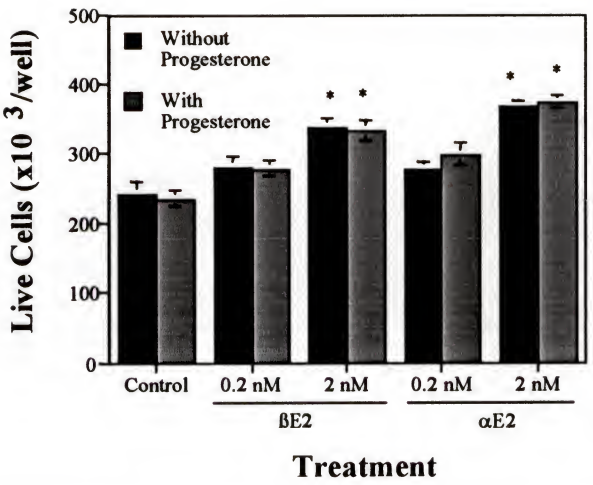


Figure 4-2. Effect of progesterone on estradiol protection of SK-N-SH cells from serum deprivation. Cells were plated at 1 x 10^6 cells per well and 48 h later cell number was determined. All wells were deprived of serum for the entire incubation period. Wells were treated with the indicated doses of βE2 or αE2 in the presence or absence of 2 nM PROG. Steroids were added concurrent with serum deprivation. *$p<0.05$ versus Control (SF) group. Depicted are mean ± sem for 6 wells per group.

PROG, respectively, and αE2 caused a 204% and a 206% protection in the presence and absence of 2 nM PROG, respectively (data not shown).

A 2 nM concentration of CORT reduced the viability of serum-deprived SK-N-SH cells by an average of 24% as compared to SF controls (Figure 4-3). This effect was not statistically significant but the trend was replicated in five different studies. CORT (2 nM) reduced the neuroprotective effect of 2 nM βE2 and αE2 by 25% and 21%, respectively (Figure 4-3).

Discussion

A 2 nM dose of either βE2 or the weak estrogen, αE2, protected SK-N-SH cells from serum-deprivation toxicity whereas neither CORT nor PROG exerted protective effects in this model with concentrations up to 200 nM. Both PROG and CORT showed a

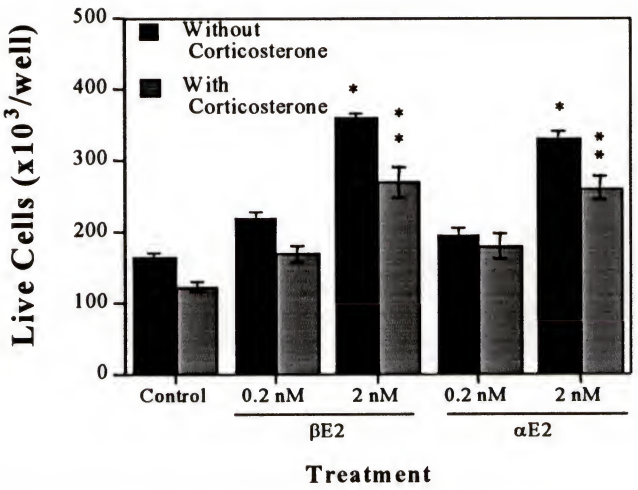


Figure 4-3. Effect of corticosterone on estradiol protection of SK-N-SH cells from serum deprivation. Cells were plated at 1 x 10^6 cells per well and 48 h later cell number was determined. All wells were deprived of serum for the entire incubation period. Wells were treated with the indicated doses of βE2 or αE2 in the presence or absence of 2 nM CORT. Steroids were added concurrent with serum deprivation. *$p<0.05$ versus Control (SF) group. **$p<0.05$ versus Control group and the corresponding treatment without CORT. Depicted are mean ± sem for 6 wells per group.

slight trend toward exacerbating neuronal death (Figures 4-1, 4-2, 4-3, Table 3-2). PROG treatment concurrent with estradiol exposure did not alter the neuroprotective effects of either βE2 or αE2. However, CORT treatment concurrent with estradiol treatment significantly reduced the protection conferred by either estradiol isomer.

The lack of a significant PROG effect on SK-N-SH survival under conditions of serum deprivation suggests that the neuroprotective effects of progestins previously reported may be insult-dependent. Neuroprotective effects of PROG have been observed in *in vivo* models of ischemia (Roof et al. 1993; Jiang et al. 1996; Gonzalez-Vidal et al. 1998), traumatic brain injury (Roof et al. 1997), and in some cell culture models of excitotoxicity (Goodman et al. 1996). However, PROG was reported not to alter cell viability when neurons were faced with oxidative challenges (Behl et al. 1995; Goodman

et al. 1996). Neuronal death in ischemia and traumatic brain injury are thought to occur by excitotoxic mechanisms (for review see Lipton and Rosenberg 1994). PROG can decrease neuronal excitability by potentiation of GABA-mediated chloride currents (Gee 1988) and by attenuation of NMDA responses (Smith et al. 1987). Either of these effects could mediate protection from excitotoxic insults, as $GABA_A$ receptor potentiation (Green and Cross 1994) and NMDA receptor inhibition (George et al. 1988; Park et al. 1988) have been shown to attenuate excitotoxic cell death.

In these studies, CORT exhibited about a 25% decrease in the survival of serum-deprived SK-N-SH neuroblastoma cells. CORT also attenuated the survival of serum-deprived SK-N-SH cells by 20 to 25% in the presence of either isomer of estradiol. The doses of CORT used here are in the range of those previously shown to enhance neuronal vulnerability to a wide variety of insults (Sapolsky et al. 1988). The magnitude of the CORT enhancement of toxicity shows dependency on the toxin and the cell-type used (Sapolsky et al. 1988). CORT is hypothesized to increase neuronal vulnerability by impairment of energy metabolism (Sapolsky 1994). This may be due to inhibition of glucose transport into neurons (Horner et al. 1990). Further, it has been shown that CORT exacerbation of toxicity can be blocked by increasing glucose concentrations (Sapolsky et al. 1988; Behl et al. 1997a). Interestingly, estrogen has been shown to increase brain glucose uptake in ovariectomized rats (Bishop and Simpkins 1995). Whether either of these effects occur in SK-N-SH cells remains to be determined.

Estrogen may also protect neurons through an antioxidant mechanism (Hall et al. 1991; Behl et al. 1995; Goodman et al. 1996). *In vitro*, βE2 inhibits lipid peroxidation with a potency similar to that of α-tocopherol (Nakano et al. 1987; Sugioka et al. 1987;

Mukai et al. 1990; Hall et al. 1991). Interestingly, CORT exhibits mild pro-oxidant activities in these *in vitro* assays of lipid peroxidation (Mooradin 1993). Additionally, βE2 decreases and CORT increases lipid peroxidation of cultured hippocampal neurons due to glutamate and Aβ toxicity (Goodman et al. 1996). PROG has been shown to decrease the lipid peroxidation associated with excitotoxic insults (Goodman et al. 1996) but does not exert any antioxidant capacity in cell-free assay (Mooradin 1993). This suggests that PROG decreases oxidative damage in that paradigm by attenuating the insult upstream of free radical generation perhaps by attenuating NMDA-receptor activation as described above.

The concentration of βE2 (2 nM) used in this study is near the peak physiological plasma concentrations seen in the normal menstrual cycle (Baird and Guevara 1969; Mishell et al. 1971). A more physiological dose (0.2 nM) of estradiol has previously been shown to exert significant neuroprotection in this model; however, the higher dose was chosen for these studies to increase the likelihood that both increases and decreases in estradiol-induced protection could be observed.

Plasma concentrations of PROG range from about 2 nM during the follicular phase of the menstrual cycle up to about 60 nM during the luteal phase (Tagatz and Gurpide 1973). The doses of PROG used in this study covered this concentration range. We utilized the 2 nM dose of PROG to determine interactive effects with estradiol, as this dose of PROG previously showed an exacerbation of serum-deprivation toxicity (Figure 4-1, Table 3-2).

Estrogens are currently under investigation as potential treatments for the neurodegeneration seen in AD. As estrogen therapy is generally given with concurrent

progestin therapy to alleviate the risk of endometrial cancer (Grady et al. 1992), it is important to understand the effects of progestins on the neuroprotective effects of estrogens. These results suggest that progestins concurrent with ERT will not alter the neuroprotective effects of estrogens. Further, the increased concentrations of glucocorticoids reported in AD patients may attenuate the protective actions of estrogens.

CHAPTER 5
STRUCTURE-ACTIVITY RELATIONSHIP FOR THE NEUROPROTECTIVE EFFECTS OF STEROIDS

Introduction

Postmenopausal ERT is associated with a 40% reduction in the incidence of AD (Paganini-Hill and Henderson 1994) and a delay in the onset of the disease (Tang et al. 1996). Further, ERT has been reported to improve cognitive function in Alzheimer's patients in small, open clinical trials (Fillit et al. 1986; Honjo et al. 1989; Ohkura et al. 1994). Estrogens may also be important in protecting patients from more acute brain damage, as ERT reduces the incidence of and mortality from stroke in post-menopausal subjects in some (Paganini-Hill et al. 1988; Falkeborn et al. 1983; Finucane et al. 1993) but not all studies (Boysen et al. 1988). We have recently demonstrated that βE2 exerts neuroprotective effects at pM concentrations in both animal and tissue culture models (Bishop and Simpkins 1994; Green et al. 1997a; Singh et al. 1994; Singh et al. 1995; Chapter 3).

The SAR of estrogenic actions mediated by both known forms of the ER are well delineated. βE2 is the model ligand of the ERs and binds to both receptors with a K_d of about 0.3 nM (Sandborne et al. 1971; Kuiper et al. 1996; Anstead et al. 1997; Kuiper et al. 1997; Tong et al. 1997; Tremblay et al. 1997). This high-affinity interaction requires a cyclopentaphenanthrene structure (shown in Figure 1-3), a phenolic A ring with a hydoxy function at the 3 position, and a hydroxy or other hydrogen-bond accepting group at the 17β-position (Anstead et al. 1997). The estrogenic potency of a steroid is determined by

the affinity for ER binding as well as the affinity of the steroid-ER complex to the ERE. Several estrogens, such as αE2 and estriol, are weaker estrogens than their binding affinities for either ER subtype would suggest (Clark et al. 1982; Klinge et al. 1992; Wiese et al. 1997).

The aforementioned neuroprotective effects of estradiol appear to be independent of the estrogenic potency of the molecule since the weak estrogen, αE2, was as effective as βE2 in protecting neuronal cells from serum-deprivation, Aβ-induced toxicity, and EAA exposure (Green et al. 1996; Behl et al. 1997b; Green et al. 1997a; Zaulynov et al. 1999; Chapter 3). Several studies have correlated βE2-mediated neuroprotection with antioxidant activity of the steroid (Hall et al. 1991; Behl et al. 1995; Goodman et al. 1996). αE2 and βE2 show equal potency in their antioxidant activity (Mooradian 1993; Blum-Degen et al. 1998).

If estrogens are exerting their neuroprotective effects by an antioxidant mechanism, βE2 should attenuate the oxidative stress-induced activation of NFκB. The transcription factor NFκB is directly activated by intracellular peroxides (Schreck et al. 1991; Schmidt et al. 1995) and in addition, a number of toxic insults which are associated with oxidative stress, including Aβ toxicity (Lezoualc'h and Behl 1997; Kaltschmidt et al. 1997), EAA exposure (Kaltschmidt et al. 1995; Grilli et al. 1996) and haloperidol toxicity (Post et al. 1998). Compounds with antioxidant activity block the toxin-induced activation of NFκB (Meyer et al. 1993; Grilli et al. 1996; Lezoualc'h et al. 1998a; Post et al. 1998).

The purpose of this study was to elucidate the SAR for the neuroprotective effects of estrogens using a model of neurotoxicity based upon the survival of SK-N-SH cells

under conditions of serum deprivation. Further, the effect of estradiol on H_2O_2-induced NFκB activation was assessed as a marker for antioxidant activity of the steroid.

Materials and Methods

Experimental Media

Experiments were initiated by plating SK-N-SH cells at a density of 1 x 10^6 cells/ml with 1 ml/well in 24 well plates or 0.1 ml/well in 96 well plates. In all studies, cells were cultured in RPMI-1640 (SF group), RPMI-1640 media supplemented with 10% FBS (FBS group), or SF RPMI-1640 supplemented with one of the following compounds at a concentration of 2 nM: βE2; αE2; 17β-estradiol 3-O-methyl ether; 17α-estradiol 3-O-acetate; estrone; estrone-3-O-methyl ether; estriol; estriol-3-O-methyl ether; 17α-ethynyl estradiol; 17α-ethynyl estradiol 3-O-methyl ether; 2-hydroxy estradiol, 2,3-methoxyestradiol; estratrien-3-ol (E-3-ol); pregnisolone; methylprednisolone; DES; DES mono-O-methyl ether; DES di-O-methyl ether; aldosterone; [2*S*-(2a, 4aα, 10aβ)]-1,2,3,4,4a,9,10,10a-octahydro-7-hydroxy-2-methyl-2-phenanthrenemethanol (PAM); [2*S*-(2a, 4aα, 10aβ)]-1,2,3,4,4a,9,10,10a-octahydro-7-hydroxy-2-methyl-2-phenanthrenecarboxaldehyde (PACA); phenol; 5,6,7,8 tetrahydronapthol; butylated hydroxytoluene; or butylated hydroxyanisol (See appendix for structures of compounds). All steroids are from Steraloids, Inc., Wilton, NH. PAM and PACA were a generous gift of Dr. Doug Covey, Washington University, St. Louis, MO, and other chemicals were obtained from Sigma Chemical Co., St Louis, MO. Compounds were initially dissolved in absolute ethanol and diluted in RPMI-1640 media to a final concentration of 2 nM unless otherwise indicated. To control for possible ethanol effects in the treated wells, both the

SF media (SF group) and the FBS media (FBS group) were supplemented with 0.0001% V/V absolute ethanol. In these studies, the FBS groups and SF groups averaged 7.08 ± 0.37 x 10^5 and 2.11 ± 0.20 x 10^5 cells/well, respectively.

Quantitation of Cell Viability

Cell viability was assessed at 48 h of treatment using the trypan dye-exclusion method or the MTS dye conversion assay as described in Chapter 2.

Estrogen Receptor Binding

Whole cell specific ^{3}H-estradiol binding was assessed as described in Chapter 2. MCF-7 cells served as a positive control in each replicate.

NFκB Electromobility Shift Assay

SK-N-SH cells were grown to approximately 75% confluence in Corning 100 mm dishes. αE2 or βE2 were added to cultures at the indicated concentrations 2 h prior to addition of 150 μM H_2O_2. Control dishes were treated with 0.01% absolute ethanol. Following 1 h exposure to H_2O_2, nuclear protein was extracted and analyzed by EMSA as described in chapter 2. All dishes were stained with calcein AM/propidium iodide and photographed to verify confluency and viability. Calcein AM/propidium iodide staining did not alter NFκB activity.

Southwestern Analysis

Southwestern analysis was performed as described by Gai et al. (1997). 20 μg of nuclear protein was separated on by SDS-PAGE in a Biorad Mini Protean gel electrophoresis apparatus. The gel was rinsed, and then renatured for 3 h in renaturation buffer (50 mM NaCl, 2 mM EDTA, 0.1 mM DTT, 4 mM urea, 10 mM Tris, pH 7.4). The

renatured gel was blocked in blocking buffer (50 mM NaCl, 2 mM MgCl2, 1 mM mercaptoethanol, 5% nonfat dry milk, 10 mM Tris, pH 8.0) for 2 h. ^{32}P-labeled double-stranded NFκB oligonucleotide (100,000 cpm), and 200 μg of poly(dI.dC) was added to the blocking buffer and the gel incubated for an additional 2 h. The gel was washed three times for 10 min in blocking buffer, dried and exposed to radiographic film overnight. A molecular weight (MW) ladder was included on each gel. The Southwestern detected a single band at 65 KD which is the MW of the DNA binding subunit of NFκB. Southwestern analysis was preformed with the technical assistance of Eileen Monck.

Statistical Analysis

The significance of differences among groups was determined by one way ANOVA. Planned comparisons between groups were performed using Scheffe's F-test. All statistical analysis was performed on raw data, and $p<0.05$ was considered significant.

Results

Estratriene Structure and Survival of Serum-Deprived SK-N-SH Cells

βE2 caused a dose-dependent protection of serum-deprived SK-N-SH cells with significant neuroprotection at the 0.2 nM dose (Figure 5-1). This effect was robust with the 2 nM concentration of βE2 showing neuroprotection in nine separate trials (Figures 5-1 to 5-4 and Tables 5-1 to 5-3). αE2, E-3-ol, and 2-hydroxy-estradiol were equivalent to βE2 in the degree of neuroprotectivity (Figure 5-2). Estrone, estriol, and 17α-ethynyl estradiol were significantly neuroprotective but appeared to be less active than βE2

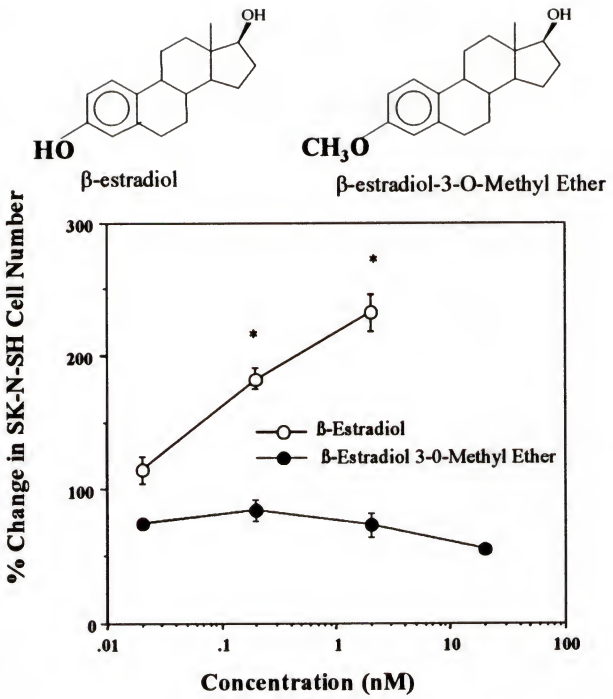


Figure 5-1. The hydroxyl function in the C3 position is necessary for neuroprotective activity as β-estradiol, but not its 3-O-methyl cogener, protects SK-N-SH cells from the toxic effects of serum deprivation. Steroids or vehicle controls were added concurrent with the insult and live cell number was determined 48 h later. Raw data were compared to the respective SF control group by ANOVA and Scheffe's F test. Data were then normalized to the SF group (=100%). *=$p<0.05$ versus SF controls. Data are expressed as mean ± SEM for 4 to 6 wells per group. The structures shown are βE2 and 17β-estradiol-3-O-methyl ether, respectively.

(Figure 5-2). However, the protection conferred by these three compounds was not statistically different from the protection conferred by βE2. The diphenolic estrogen mimic, diethylstilbesterol (DES) was an active neuroprotectant and retained nearly full neuroprotectivity when one but not both of the phenolic hydroxyl functions were replaced with an O-methyl ether function (Figure 5-3). Similarly all steroids were rendered inactive when the 3-hydroxyl group was replaced with an O-methyl ether group (Figures 5-1 and 5-2), a substitution that eliminates the acidic hydrophilic properties of the A ring. The 3-O-methyl ether of βE2 was inactive even at concentrations as high as 20 nM (Figure 5-1). However, the two three-ring compounds, PAM and PACA, which contain a phenolic A

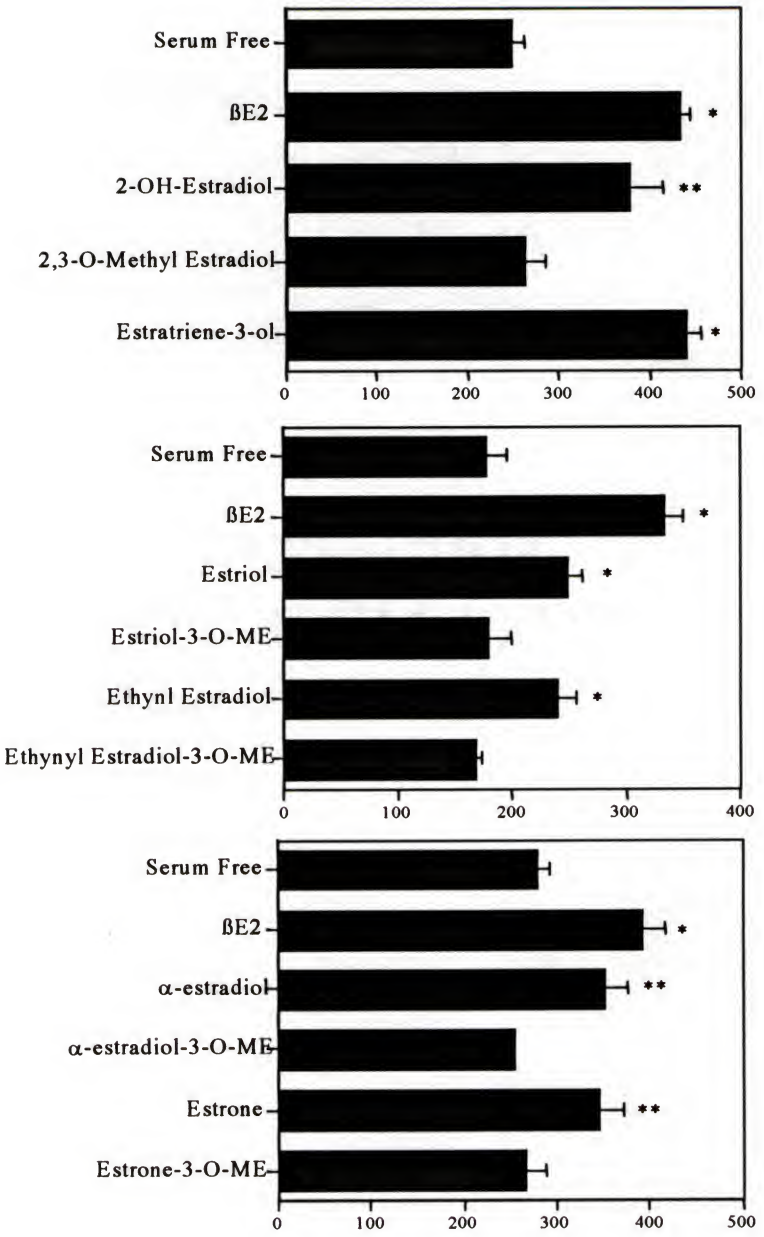


Figure 5-2. Phenolic A ring estrogens but not their 3-O-conjugates protect SK-N-SH cells from the toxic effects of serum deprivation. Steroids or vehicle controls were added concurrent with the insult, and live cell number was determined 48 h later. *=$p<0.05$ versus SF controls. **=$p<0.05$ versus SF controls and the respective 3-O-conjugate. Data are expressed as mean ± SEM for 5 wells per group. The three panels represent three individual experiments.

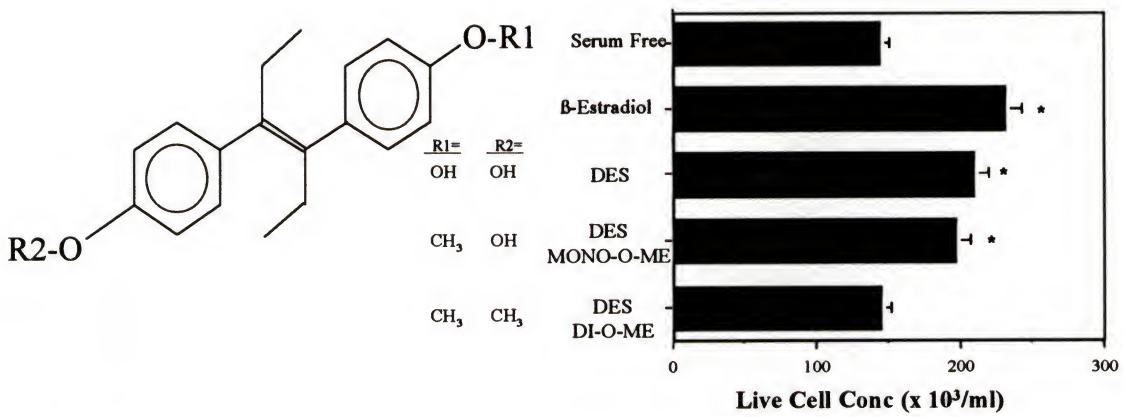


Figure 5-3. Diethylstilbesterol and DES Mono-O-methyl ether but not DES di-O-methyl ether protect SK-N-SH cells from the toxic effects of serum deprivation. Steroids or vehicle controls were added concurrent with the insult, and live cell number was determined 48 h later. *=$p<0.05$ versus SF controls and DES di-O-ME ether groups. Data are expressed as mean ± SEM for 6 wells per group. Shown is the core structure of DES and its ether conjugates.

ring but do not contain the D ring of the steroid structure were as neuroprotective as βE2 (Figure 5-4). Further, immobilization of the steroid by BSA conjugation also abolished neuroprotective effects as β-estradiol-17-hemiacetate:BSA was not significantly protective at concentrations as high as 200 nM (Table 5-1).

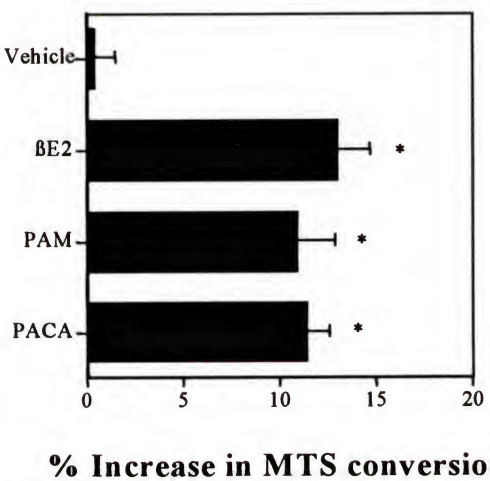


Figure 5-4. Both PAM and PACA, representing the A, B, and C rings of estradiol and estrone structure, respectively, protect SK-N-SH cells from the toxic effects of serum deprivation. The compounds or vehicle were added concurrent with insult, and MTS conversion determined 48 h later. Data are expressed as mean ± sem for 14-22 wells per group.

Table 5-1. Effect of β-Estradiol-17-Hemiacetate:BSA on Live SK-N-SH Cell Number under Serum-Free Conditions.

Treatment	Live Cell Number (Mean±SEM X 10^3/ml)
SF Controls	153 ± 12
βE2 (2 nM)	314 ± 23*
βE2:BSA (2 nM)	171 ± 15
βE2:BSA (20 nM)	206 ± 12
βE2:BSA (200 nM)	162 ± 13

*$p<0.05$ vs serum-free control groups.

Table 5-2. Effects of a Variety of Non-Phenolic A Ring Steroids on Live SK-N-SH Cell Number under Serum-Free Conditions.

Treatment	Live Cell Number (Mean±SEM X 10^3/ml)
SF Controls	177 ± 18
βE2	329 ± 33*
Prednisolone	187 ± 16
6α-Methylprednisolone	173 ± 14
Aldosterone	132 ± 18

*$p<0.05$ vs serum-free control groups.

Non-Estratriene Steroids and Survival of Serum-Deprived SK-N-SH Cells

Several steroids were found to lack neuroprotective activity at a 2 nM concentration in Chapter 3 (Table 3-2). In this study, an additional three pregnane derivatives were evaluated (Table 5-2). Prednisolone, 6-methyl-prednisolone, and aldosterone showed no neuroprotective activity at a 2 nM concentration.

Non-Steroid Phenols and Survival of Serum-Deprived SK-N-SH Cells

Phenol and 5,6,7,8 tetrahydronapthol which represent the A and AB ring of the estratrien structure, respectively, were evaluated for neuroprotective activity. Phenol (2 nM) was toxic, resulting in a 22% decrease in live cells from the SF group (data not shown). Tetrahydronapthol (2 nM) had no effect on live cell number (data not shown). Finally, we assessed the activity of two lipophilic phenols, butylated hydroxytoluene and

butylated hydroxyanisol, which resulted in a 14% and a 10% decrease in live cell number from the SF group (Table 5-3).

Table 5-3. Effects of Lipophilic Phenols on Live SK-N-SH Cell Number under Serum-Free Conditions.

Treatment	Live Cell Number (Mean±SEM X 10^3/ml)
SF Controls	203 ± 16
βE2 (2 nM)	373 ± 11*
Butylated Hydroxytoluene	175 ± 11
Butylated Hydroxyanisole	182 ± 16

*$p<0.05$ vs serum-free control groups. All compounds were tested at a 2 nM concentration.

Estradiol and H_2O_2-induced NFκB Activity

Exposure to 150 μM H_2O_2 for 1 h resulted in a reproducible increase in NFκB DNA binding activity (Figure 5-5 and 5-6). Exposure to this dose of H_2O_2 for 1 h did not alter SK-N-SH viability (data not shown) although a 24 h treatment with this dose of H_2O_2 results in a 91 to 99% reduction in SK-N-SH viability (data not shown). Neither αE2 (10 μM) nor βE2 (1 nM to 10 μM) alone altered NFκB activity with treatment times of 1h (data not shown) or 3 h (Figure 5-5 and 5-6). However, 10 μM of either estradiol isomer almost completely attenuated the H_2O_2-induced increase in NFκB activity (Figure 5-5). Attenuation of H_2O_2-induced NFκB activity by βE2 appeared dose-dependent and was seen with 1 nM of βE2 (Figure 5-6).

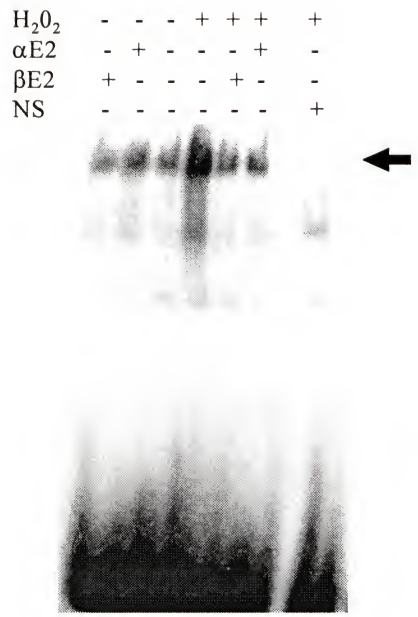


Figure 5-5. Both β-estradiol and α-estradiol attenuate the H_2O_2-induced activation of NFκB. Either αE2 or βE2 (10 μM) were added 2 h prior to 150 μM H_2O_2 and NFκB DNA binding activity was determined in the nuclear extracts. NS indicates the addition of 100-fold excess of cold oligonucleotide. The black arrow indicates the specific binding band. The gel shown is representative of 6 repetitions.

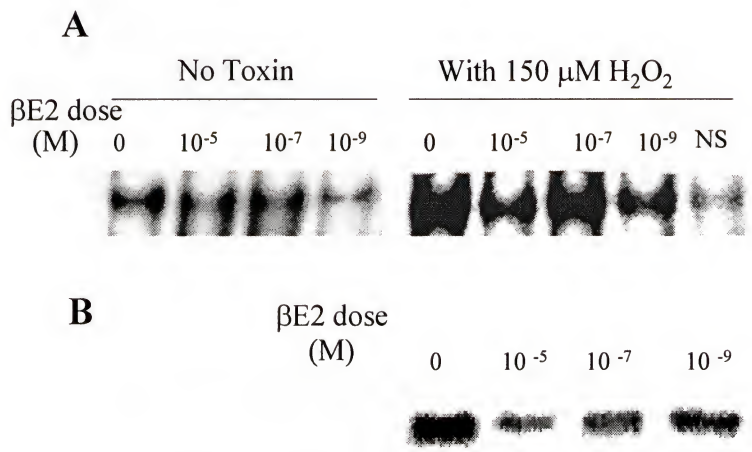


Figure 5-6. Dose-dependent attenuation of H_2O_2-induced NFκB activity by β-estradiol in SK-N-SH cells. The indicated dose of βE2 was added to SK-N-SH cells 2 h before 150 μM H_2O_2, and nuclear extracts were analyzed for NFκB DNA binding activity by EMSA analysis (A) or Southwestern analysis (B). A 100-fold excess of unlabeled oligonucleotide was added to the NS lane. The gels shown are representative of 3 repetitions.

ER Binding in SK-N-SH cells

Finally, we evaluated the SK-N-SH cells for the presence of specific ^{3}H-βE2 binding in whole cell preparations. SK-N-SH cells did not demonstrate specific binding with only 1 ± 0.9 fmol of ^{3}H-βE2 bound per 10^{6} cells as compared to the estrogen-receptor containing MCF-7 cells with 56 ± 6.5 fmol specific binding per 10^{6} cells ($p<0.05$ vs non-specific binding tubes) using this binding protocol.

Discussion

This study represents the first demonstration of the SAR for neuroprotection associated with low doses of neuroprotective steroids. A hydroxyl function on the aromatic ring of estratrienes is required for their neuroprotectivity. However, a phenolic ring is not neuroprotective in the absence of a steroid-like structure. Simple enhancement of the lipophilicity of the phenolic group was not sufficient to impart neuroprotectivity to this moiety, as two highly lipophilic phenols, butylated hydroxytoluene and butylated hydroxyanisole were both inactive at the low nM concentrations used in our assay. The conformational shape of the flat, phenolic ring and/or the enhanced acidity of phenols relative to cyclohexanols may be important in conferring the observed neuroprotective activity. However, it is clear from these data that a steroid structure is inactive in the absence of a phenolic A ring, as is a phenolic ring in the absence of at least three rings of the steroid backbone.

Two 19-carbon androgens and five 21-carbon steroids were evaluated for neuroprotection in our assay (Table 3-2 and Table 5-2). The two androgens containing a C-3 ketone, namely testosterone with a partially unsaturated A ring and

dihydrotestosterone with a saturated A ring, were both inactive. Similarly, all five of the 21-carbon pregnane derivatives that were tested contained a C-3 ketone function; the three Δ^4-steroids -corticosterone, progesterone, and aldosterone- and two $\Delta^{1,4}$-steroids - prednisolone and 6-methylprednisolone- were inactive (Table 3-2 and 5-2). Finally, cholesterol was tested because it has a 3-hydroxyl function on a completely saturated A ring and was inactive (Table 3-2). This indicates that the 3-hydroxyl function present on a steroid backbone is not sufficient for low-dose neuroprotective activity in the absence of the aromatic A-ring.

Immobilization of the estrogen molecule by conjugation to a BSA molecule attenuated the protective efficacy of the steroid. We evaluated the 17-conjugate of E2:BSA rather than the more commonly used 6-conjugate in order to decrease interference of the BSA with the 3-OH group. Additionally, the 6-conjugated βE2:BSA was evaluated at estradiol concentrations as high as 20 μM in another neuronal cell line, HT-22 cells, and no protection was observed from Aβ toxicity (Green et. al. 1997c; Table 6-1). The BSA conjugation allows exposure of the extracellular space to the steroid but prohibits the diffusion of the estradiol to the intracellular spaces. E2:BSA has been shown to induce phosphorylation of ERK in this cell line (Watters et al. 1997) suggesting that the neuroprotective effects of estradiol may not be mediated by estrogen-induced ERK phosphorylation. The lack of neuroprotection by the estradiol:BSA conjugates indicates that the estrogen must be free to pass into or through the neuronal membranes to have neuroprotective actions.

The present study provides evidence for the dissociation of the estrogenic potency of steroids and their neuroprotectivity. Several of the active neuroprotective phenolic A

ring steroids, including αE2, estriol, 2-hydroxy-estradiol, and E-3-ol have only weak or no estrogenic activity as assessed by binding to the estrogen receptor, binding of the steroid-ER complex to the ERE, or the mitotic response to acute or chronic exposure of uterine and mammary tissue to the steroid (Huggins et al. 1954; Koreman 1969; Martucci and Fishman 1979; Clark et al. 1982; Clark and Mardaverich 1983; Papendorp et al. 1985; Anstead et al. 1997; Wiese et al. 1997). Further, the neuroprotective efficacy of these estrogens is not explained by the recent discovery of ERβ, since αE2, estriol, and estra-2,17β-triol have similarly low binding affinities to ERβ (Kuiper et al. 1997), and are as neuroprotective as βE2 in our assay system. This observation suggests that the neuroprotectivity of these compounds is not mediated primarily through the known nuclear ER. This conclusion is consistent both with the observation that neuroprotectivity is preserved even in the face of a classical estrogen antagonist (Green et al. 1997a; Figure 3-3) and the lack of specific estradiol binding sites in these cells. Additionally, the neuroprotective activity of βE2 has been demonstrated in HT-22 cells, which lack a functional ER as determined by reporter gene assays (Behl et. al. 1995).

Behl et. al. (1997b) has recently reported a similar SAR for estrogen protection of the hippocampal neurons from oxidative damage. Those studies required a minimum dose of 10 μM to achieve significant neuroprotection, whereas we demonstrate significant protection of SK-N-SH cells with doses as low as 0.2 nM from either serum-deprivation (Green et al. 1997a; Figures 3-1 and 3-2) or Aβ toxicity (Green et al. 1996). This 50,000 fold difference in effective doses may be due, in part, to different properties of the cell lines, different culturing conditions or different toxic insults. However, the low nM

estradiol doses at which we demonstrate neuroprotection *in vitro* also appear to be capable of neuroprotection *in vivo* using a rat focal ischemia model (Shi et al. 1997).

Phenolic A ring steroid compounds may exert neuroprotective actions through an antioxidant mechanism. Lipophilic phenols are well known to be antioxidants (Niki 1987). Estrogens, specifically phenolic A ring estratrienes, have been shown to have antioxidant activity and to reduce membrane lipid peroxidation (Nakano et al. 1987; Mukai et al. 1990; Hall et al. 1991). Also, βE2 protects neurons from oxidative insults such as hydrogen peroxide (Behl et al. 1995), iron sulfate (Goodman et al. 1996), and Aβ peptide (Behl et al. 1995; Goodman et al. 1996; Green et al. 1996) toxicity. This is significant, as increased lipid peroxidation is associated with a variety of neurodegenerative diseases, including ischemic/anoxic insults (Tappel 1973; Flamm et al. 1978; Yoshida et al. 1982; Braughler and Hall 1989) and Alzheimer's disease (Carney et al. 1991; Smith et al. 1991; Hensley et al. 1994).

The ability of estradiol to block the H_2O_2-induced activation of NFκB in SK-N-SH cells is further suggestive of an antioxidant mechanism of action in this cell line. However, estradiol may interfere with NFκB activation by other mechanisms. Sun et al. (1998) have reported that βE2 can modulate IκBα protein levels in Hela cells. It is unlikely that estradiol attenuation of H_2O_2-induced NFκB activity in these SK-N-SH studies is mediated by upregulation of IκBα expression, as no increase in IκBα levels was observed after 3 h exposure to βE2 (Green and Simpkins, unpublished observations).

Although the structural requirement for estratriene neuroprotection is suggestive of an antioxidant mechanism for estrogens, the doses with which we show neuroprotection complicates that view. Antioxidant actions of estrogens generally require

low μM doses of the steroid (Nakano, et al. 1987; Mukai et al. 1990; Hall et al. 1991; Goodman et al. 1996; Behl et al. 1997b); however, we achieved neuroprotection with as low as 0.2 nM of the steroid. Other potent antioxidant compounds were inactive at the low doses used in our assay system, including methylprednisolone, a neuroprotective steroid which inhibits lipid peroxidation at high μM concentrations (Hall 1992), and two phenol with antioxidant properties, butylated hydroxytoluene and butylated hydroxyanisol. However, concentrations of βE2 as low as 1 nM are capable of abating the H_2O_2-induced increase in NFκB activity suggesting that lower doses of βE2 may be capable of exerting antioxidant activity in these cells. Similarly, our laboratory has recently demonstrated a decrease in Aβ-associated lipid peroxidation with similar doses of βE2 in SK-N-SH cells (Gridley et al. 1997). The low estradiol concentrations with which we demonstrate both neuroprotective and antioxidant activity in these cell imply that estrogens may be interacting with some component of this system to increase both the neuroprotective and antioxidant potency of βE2.

We tested compounds at a 2 nM dose, a dose 10-fold higher than necessary to see the neuroprotective effects of βE2 and αE2 in our assay system (Green et al. 1997a). This dose is physiologically relevant, only slightly above the peak levels of circulating estrogens in pre-menopausal women. The inactivity of the non-phenolic estrogens was not due to the dose tested, as concentrations as high as 20 μM of 17β-estradiol-3-O-methyl ether showed no neuroprotective activity (Figure 5-1). Similarly, corticosterone and progesterone at concentrations up to 200 nM were not neuroprotective in our assay system (Figure 4-1). However, it is possible that one or more of the compounds tested may show neuroprotective efficacy at higher concentrations.

NFκB is intimately involved in modulating neuronal viability and is reported to mediate both neurotoxicity and neuroprotection, depending on the system (for review see Lipton 1997). It is unclear if diminution of toxin-induced NFκB activity contributes to or is the result of the neuroprotective effects of estrogens; however, blockade of NFκB activity has been shown to reduce, but not eliminate, the toxic effects of intracellular hydroxides (Grilli et al. 1996; Post et al. 1998). Irrespective of a causal role in neuroprotection, estrogen abatement of NFκB activation may play an important role in the pathophysiology of AD, as the expression of several components of senile plaques in AD is positively regulated by NFκB, including APP (Grilli et al. 1995; Yan et al. 1995), α1-antichymotrypsin (Lieb et al. 1996), and IL6 (Ray et al. 1988).

The phenolic A ring requirement for the neuroprotective action of estrogens suggest that these steroids may protect neurons though a mechanism that does not require induction of classical ER-mediated transcription. Phenolic A ring estrogens which lack significant estrogenic side effects may be useful in the treatment of chronic neurodegenerative diseases and acute neuronal injury which are presently untreatable. The phenolic A ring requirement for the neuroprotective action of estrogens may provide important clues to their mechanism of action and for the design of drugs with enhanced neuroprotective properties.

CHAPTER 6
ESTROGEN RECEPTOR-INDEPENDENT CYTOPROTECTION BY ESTRATRIENES

Introduction

Recent evidence suggests a role of ovarian steroids in normal maintenance of brain function and suggests that a loss of these hormones at menopause may play a role in the neurodegeneration associated with AD. Post-menopausal ERT is associated with a 40% decline in the incidence of AD (Pagaini-Hill and Henderson; 1994) and a delay in the onset of the disease (Tang et al. 1996; Kawas et al. 1997). Further, estrogens are potent neuroprotective agents in a variety of *in vivo* model systems, including fimbria-fornix lesions (Rabbani et al. 1997) and MCA occlusion (Shi et al. 1997). Additionally, we and others have shown that estrogens protect cultured neurons and neuronal cell lines from a variety of insults, including serum-deprivation (Bishop and Simpkins 1994; Green et al. 1997a), Aβ toxicity (Behl et al. 1995; Behl et al. 1997b; Green et al. 1996), and glutamate toxicity (Behl et al. 1995; Singer et al. 1996; Zaulynov et al. 1999).

Many effects of estrogens are mediated by the binding of the steroid to its nuclear ER and the potent binding of the steroid-receptor complex to the ERE thereby activating transcriptional events. Increasingly, effects of estrogens are being described that occur by alternate mechanisms. Estrogens have direct effects on neuronal membranes that have been shown to involve specific interactions with membrane binding sites (Teyler et al. 1980). Further, estradiol potentiates excitatory post synaptic potentials in hippocampal CA1 neurons within two minutes of its addition to the slice (Wong and Moss 1991).

Estrogens have also been shown to modulate Ca^{2+} fluxes in multiple subtypes of neurons by a non-genomic mechanism (Morley et al. 1992; Nemere and Norman 1992).

Several lines of evidence are emerging to suggest that the aforementioned neuroprotective effects of estrogens are not mediated through the classical ER pathway. First, tamoxifen, a mixed ER agonist/antagonist, does not prevent estrogen's protection of serum-deprived SK-N-SH neuroblastoma cells and has no protective ability of its own (Green et al. 1997a). Second, the SAR for the neuroprotective effects of estrogens differs markedly from their SAR for binding to the ER with the result that many classically weak or inactive estrogens are potent neuroprotective agents (Behl et al. 1997b; Green et al 1997a; Green et al. 1997b). Finally, estrogen protection against oxidative stress, including Aβ toxicity, has been demonstrated in HT-22 cells, a murine hippocampal cell line that lacks ERs (Behl et al. 1995). This study required an estrogen dose of 10 μM to achieve significant neuroprotection, whereas we demonstrate protection against Aβ toxicity with physiological estrogen doses of 0.2 to 2 nM (Green et al. 1996). Work in our laboratory pointed to the GSH content of the media as the mechanism underlying the discrepancy in effective protective doses of estrogens (Green et al. 1997c; Gridley et al. 1998). The purpose of the present study is four-fold: (1) to determine if physiological doses of estrogens could protect neuronal cells in the absence of an ER; (2) to evaluate the presence of GSH in the culture media on the neuroprotective effects of estrogens in the absence of an ER; (3) to determine if neuroprotective estratrienes which interact only weakly with the ER exhibit similar interactions with GSH; and (4) to determine if estratrienes could protect red blood cells (RBCs) even though these cells lack a nucleus.

Materials and Methods

Experimental Media

Experiments were initiated by plating HT-22 cells at a concentration of 10^6 cells/well in Nunc® 24 well plates, and cells were allowed to attach for 4 h before treatment. Cells were exposed to 20 μM Aβ in the presence of the indicated dose of βE2, αE2, or E-3-ol either in RPMI media with 10% charcoal-stripped FBS containing 3.25 μM GSH or the same RPMI media without GSH. All steroids were dissolved initially at 1 mg/ml in absolute ethanol and diluted in RPMI-1640 to the final concentration indicated. To control for possible ethanol effects in the steroid-treated wells, we supplemented steroid-free groups with absolute ethanol at a concentration of 0.01% (v/v).

Quantitation of Cell Viability

Cell viability on HT22 cells was assessed at 48 hr of treatment using the trypan blue dye exclusion method as described in Chaper 2. Cell viability was also assessed by the LIVE/DEAD® assay as described in Chapter 2.

ER Binding

ER binding was carried out as described in Chapter 2.

Collection of RBCs

RBCs for this experiment were obtained by venipunture from three healthy male subjects (aged 32 to 48 yrs). Blood was drawn by K.E. Gridley, Dr. W.J. Millard or the hematology laboratory in Shands Hospital. Approximately 10 ml of whole blood were collected in hepranized tubes, and the RBCs were isolated by centrifugation at 1000 RPM

for 5 min. Cells were then washed three times with a 0.9% saline solution. After the final wash, approximately 5 ml of cells were suspended in 5 ml of 0.9% saline.

Estratriene and $FeCl_3$ treatment

0.25 ml of RBCs in 0.9% saline were incubated at 37°C for 4 h with either 0.25 ml of 0.9% saline (Control group) or 0.25 ml of 400 μM $FeCl_3$. The final concentration of $FeCl_3$ was 200 μM. βE2, αE2 or E-3-ol were added concurrent with the $FeCl_3$ at final concentrations of 0.2 to 200 nM. Control and $FeCl_3$ groups were treated with 0.001 % v/v ethanol as a vehicle control. Every 15 min during this incubation, tubes were inverted to keep the cells in suspension. At the end of the study, the number of RBCs in each sample was determined by counting in duplicate on a Neubauer hemacytometer. One of the four studies presented was performed in collaboration with K.E. Gridley.

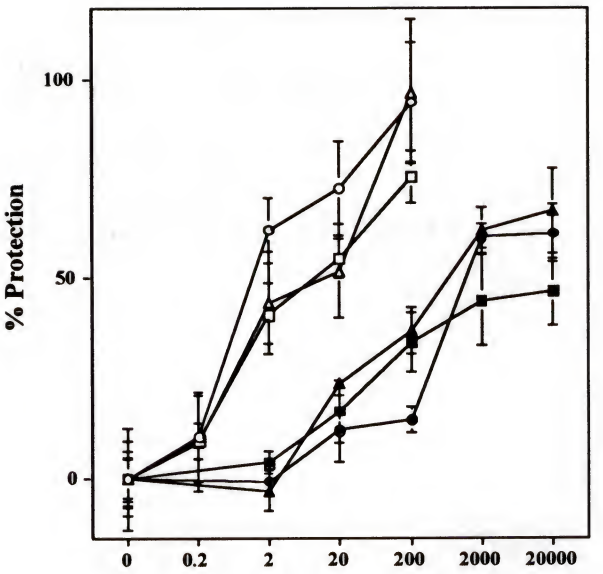
Statistical Analysis

The significance of differences among groups was determined on raw data by one-way ANOVA. Planned comparisons between groups used was done by Scheffe's F-test. For all tests, $p < 0.05$ was considered significant. ED_{50} values were log-transformed, and the resulting values were analyzed by a two way ANOVA.

Results and Discussion

Effects of Estrogens and Glutathione on Amyloid Toxicity in HT-22 Cells

We present here a synergistic interaction between neuroprotective estrogens and the protection conferred by the intracellular antioxidant GSH. βE2, the potent naturally occurring estrogen, protected HT-22 cells from cell death induced by the neurotoxic



ED_{50} Values (nM) ± SEM		
	With GSH	Without GSH
ßE2	5 ± 2	3266 ± 408
E-3-ol	13 ± 4	3683 ± 964
αE2	7 ± 2	1014 ± 156

Figure 6-1. Effects of estratrienes in the presence and absence of glutathione on the neurotoxicity induced by Aβ (25-35) in HT-22 cells. Cells were exposed to 20 μM Aβ (25-35) in the presence of the indicated dose of βE2, αE2, or E-3-ol either in media containing 3.25 μM GSH or media without GSH for 48 h prior to viability assessment. The glutathione effect was highly significant with f=364 (df=1) and $p < 0.0001$ whereas the effect of estrogen type was not significant (f=3.4, df=2) and no interaction was found between estrogen type and glutathione (f=2.47, df=2). Data were normalized to the Aβ-free control group as 100% protection and the Aβ alone group as 0% protection. The mean ± sem for 3-5 wells per group are presented in the graph and the average ED_{50} values ± sem are presented in tabular form.

amyloid fragment, Aβ (25-35) fragment with an ED_{50} of 5 nM, significant neuroprotection with 2 nM and complete protection with 200 nM of the steroid (Figure 6-1). However, this potency was dependent on the presence of 3.25 μM GSH in the culture media, as the βE2 potency was reduced 628-fold when GSH was absent from the media (Figure 6-1). Similarly, when viability was assessed by the calcein AM/propidium iodide assay, 200 nM βE2 protected about 50 % and 95 % of the cells in the absence and presence of GSH,

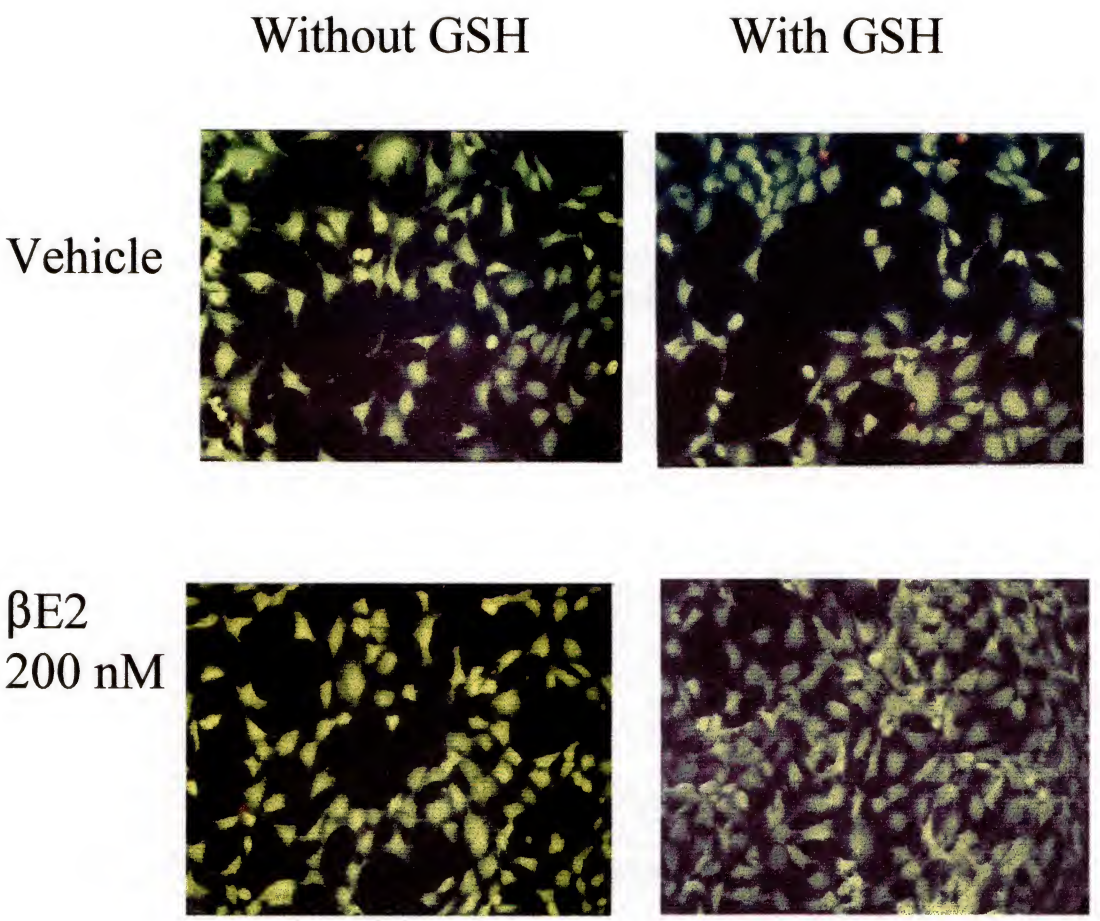


Figure 6-2. Representative photomicrographs of HT-22 cells depicting the effects of βE2 on Aβ (25-35) neurotoxicity in the presence and absence of glutathione. Cells were exposed to 20 μM Aβ 25-35 and ethanol vehicle or 200 nM βE2 either in media containing 3.25 μM GSH or media without GSH for 48 h prior to viability assessment. Live cells are stained bright green. Pictures were taken on a 200X magnification.

respectively (Figure 6-2). This estradiol-GSH interaction was also observed using Aβ (1-40), one of the forms of amyloid found in AD plaques. A 200 nM βE2 dose offered 99.9 and 35.6 % protection in the presence and absence of GSH, respectively (Figure 6-3). This GSH effect may explain the discrepancy in protective βE2 doses seen in the literature. Goodman et. al. (1996) and Behl et. al.(1995; 1997b), using culture medias lacking GSH, showed that μM doses of estradiol were required to protect primary neurons and HT-22 cells. The dose of GSH used in our studies is comparable to the low μM GSH concentrations found in the CSF (Baronti et al. 1992), suggesting that such an interaction may occur *in vivo*. This is further supported by the observation that significant neuroprotection is conferred by low nM doses of estrogens in rodent models (Rabbani et al. 1997; Shi et al. 1997).

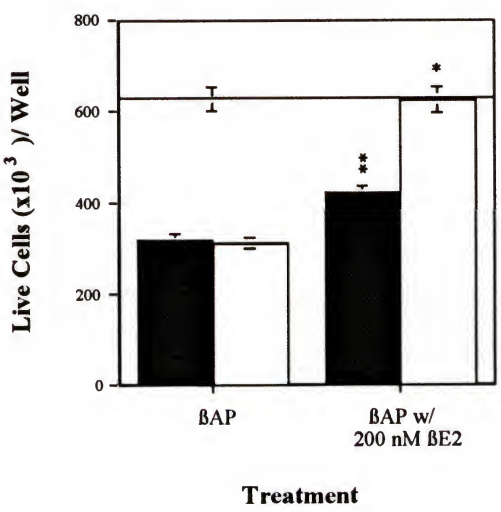


Figure 6-3. Effect of β-estradiol in the presence and absence of glutathione on the neurotoxicity induced by Aβ (1-40) in HT-22 cells. Cells were exposed to 20 μM Aβ 1-40 in the presence of 200 nM β-estradiol either in media containing 3.25 μM GSH or media without GSH for 48 h prior to viability assessment. Data were presented as mean ± sem for 3-4 wells per group. □ indicates the presence of GSH and ■ indicates the absence of GSH. The line represents the value of the Control (no Aβ) group. The data are presented as the mean ± sem for 4 wells per group. * = $p<0.05$ versus the Aβ 1-40 alone group. ** = $p<0.05$ versus both the Aβ 1-40 alone group and the control (no Aβ) group.

Green et al. (1997c) and Behl et. al.(1997b) have demonstrated that the neuroprotection by estrogens is dependent on the phenolic nature of the steroid A ring and not on the estrogenic potency. Therefore, we also evaluated the effect of GSH on the protection conferred by two classically inactive estrogens, αE2 and E-3-ol (Figure 6-1). Both of these estrogens behaved similarly to βE2 with αE2 and E-3-ol, protecting neurons with an ED_{50} of 6 nM and 14 nM, respectively, in the presence of GSH and an ED_{50} of 1014 nM and 3683 nM, respectively, in the absence of GSH (Figure 6-1). Phenolic A ring estrogens are most likely exerting their effects by an antioxidant mechanism, as the compounds have been shown to be potent inhibitors of oxidation *in vitro* (Sugioka et al. 1987). The interaction between these estrogens and GSH could be due to redox cycling between endogenous antioxidants. Such interactions are known to occur between other antioxidants such as ascorbate and GSH (Winkler et al. 1994) as well as ascorbate and α-tocopherol (Sato et al. 1993).

Table 6-1. Effects of βE2 and βE2-6-(carboxy-methyl)oxime:BSA Conjugate on Aβ (25-35)-Induced Toxicity in HT-22 Cells.

Treatment	Live Cell Number ($x10^3$ cells) ± SEM
Control (No Aβ)	427 ± 11*
Aβ (20 μM)	189 ± 11
Aβ + 0.2 μM βE2	324 ± 14*
Aβ + 20 μM βE2:BSA	216 ± 17

Cells were exposed to 20 μM Aβ (25-35) in the presence of the indicated dose of βE2 or βE2:BSA in RPMI media containing 3.25 μM GSH for 48 h before viability was determined. Data are depicted as mean ± sem for 4 wells per group. * = $p<0.05$ versus the Aβ (20 μM) group.

We tested βE2 conjugated to BSA to determine if βE2 could protect HT-22 cells from Aβ if the estrogen was restricted to the extracellular environment. Immobilization of the steroid by BSA conjugation (βE2-6-(carboxy-methyl)oxime:BSA) abolished the ability of 20 μM βE2 to protect HT-22 cells from Aβ toxicity in the presence of GSH (Table 6-1). We previously observed that BSA conjugation at the 17 position of βE2 abolished the neuroprotective ability in a human neuroblastoma cell model (Green et al. 1997b; Table 5-1). Collectively, these data suggest that the estratrienes must be to able to interact freely with the cell membrane or the intracellular space to exert their neuroprotective effects.

The dose of GSH used in these studies had no effect on Aβ-induced toxicity by itself inasmuch as Aβ (25-35) caused a 54 ± 4% and a 55 ± 3% decrease in cell viability in the absence and presence of 3.25 μM GSH, respectively (Table 6-2). However, a GSH concentration of 325 μM caused significant protection from Aβ toxicity in another cell

Table 6-2. Effects of 3.25 μM GSH on Aβ (25-35) Induced Toxicity in HT-22 Cells.

Treatment	Live Cell Number ($x10^3$ cells) ± SEM
Control (No Aβ)	634 ± 18*
Aβ (20 μM)	289 ± 25
Aβ + 3.25 μM GSH	284 ± 19

Cells were exposed to 20 μM Aβ (25-35) in the presence or absence of 3.25 μM GSH for 48 h before viability was determined. Data are depicted as mean ± sem for 4 wells per group. * = $p<0.05$ versus the Aβ (20 μM) group.

Table 6-3. Effects of βE2 and GSH on HT-22 Cell Number in the Absence of a Toxin.

Treatment	Live Cell Number ($x10^3$ cells) ± SEM
Control	545 ± 11
3.25 μM GSH	487 ± 37
0.2 μM βE2	504 ± 29
3.25 μM GSH + 0.2 μM βE2	497 ± 23

Cells were exposed to the indicated dose of βE2 and/or GSH for 48 h before viability was determined. Data are depicted as mean ± sem for 4 wells per group. Differences between groups were not statistically significant.

type, SK-N-SH neuroblastoma cells (Gridley et al. 1998). Further, exposure of HT-22 cells to 3.25 μM GSH alone, 200 nM βE2 alone, or the two in combination did not increase cell number in the absence of an insult (Table 6-3), indicating that the increase in cell number is due to protection and not to a mitogenic effect of the compounds.

We evaluated HT-22 cells for the presence of specific ^{3}H-estradiol binding in both nuclear extracts and whole cell preparations by exchange assays previously described (Miranda et al. 1996; Nakao et al. 1981). HT-22 cells did not demonstrate specific binding in either assay whereas MCF-7 cells, an ER-containing breast tumor cell line showed significant ^{3}H-estradiol binding in both whole cell and nuclear preparations (Table 6-4). This is consistent with the observation of Behl et. al. (1995) of no increase in reporter gene activity after estradiol exposure when HT-22 cells were transfected with an ERE-reporter gene construct. The protection conferred by physiological doses of estrogens on these cells indicates that a major portion of the protective effects of estrogens is independent of the ER.

Table 6-4. Specific ^{3}H-Estradiol Binding in MCF-7 and HT-22 Cells.

	Specific ^{3}H-Estradiol Binding (fmol/10^6 cells)	
	Nuclear Pellet	Whole Cell
MCF7	35 ± 2*	56 ± 6*
HT-22	0 ± 1	6 ± 4

*$p<0.05$ versus non-specific binding tubes. Shown are mean ± sem for 3 experiments performed in triplicate.

Cytoprotective Effects of Estratrienes on RBCs

The cytoprotective effects of βE2, αE2, and E-3-ol were evaluated in RBCs. A 4 h exposure to 200 μM $FeCl_3$ resulted in lysis of 35% to 44% of RBCs from three different subjects (Figure 6-4). Co-treatment with 200 nM βE2, αE2, and E-3-ol significantly

attenuated the $FeCl_3$-induced decrease in RBC number in each experiment, protecting an average of 44%, 45%, and 44% of the cells, respectively (Figure 6-4). As RBCs are anuclear, these results portend that a nucleus is not required for a cytoprotective effect of estrogens. Further, this protection is not dependent upon the estrogenicity of the steroid, as two weak estrogens, αE2 and E-3-ol, are equally as potent and efficacious as βE2.

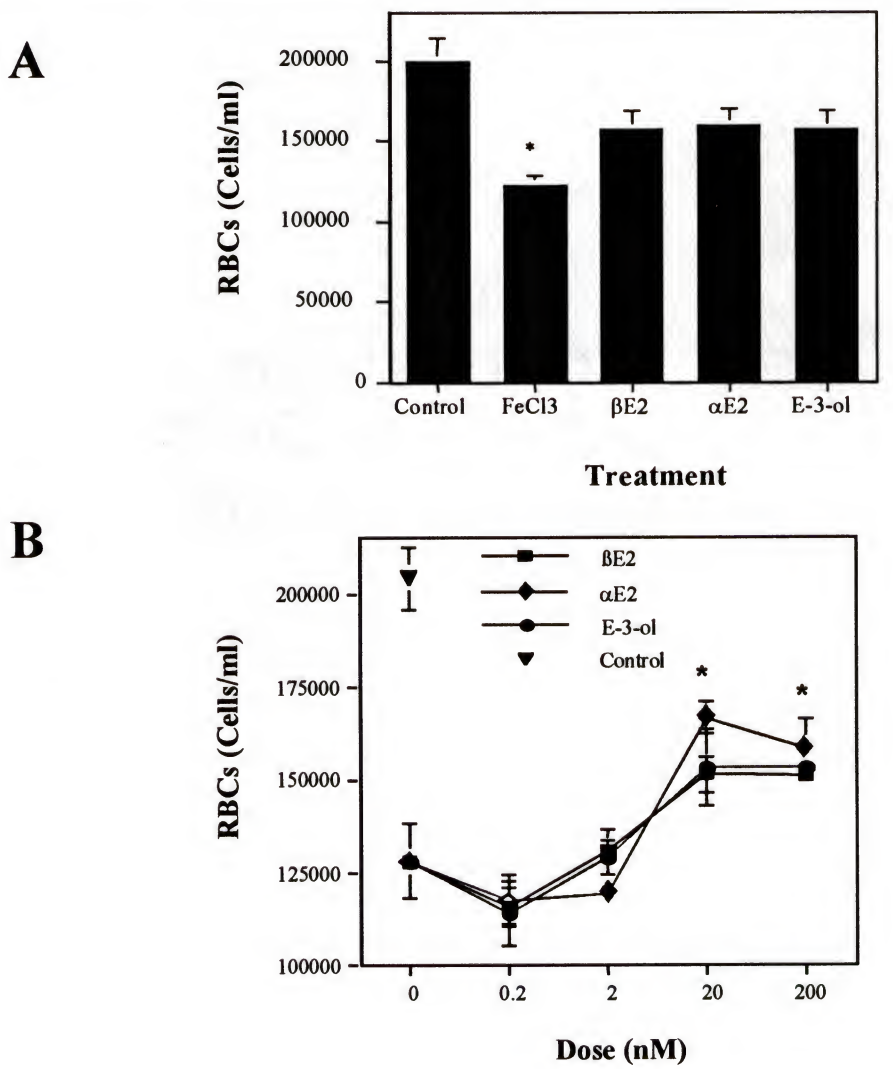


Figure 6-4. Estratrienes attenuate $FeCl_3$-induced toxicity in red blood cells. Cells were exposed to 200 μM $FeCl_3$ in the presence of 200 nM (A) or the indicated dose (B) of either βE2, αE2, or E-3-ol. (A) depicts mean ± sem for 3 individual experiments each from a different male subject with 3 to 5 replicates in each experiment, and *$p<0.05$ versus Control (no treatment) group. (B) depicts mean ± sem for 3-6 samples, and *$p<0.05$ versus 0 nM dose.

Estrogen-mediated protection of RBCs required 10- to 100-fold higher doses than estrogen-mediated protection of neuronal cells (Bishop and Simpkins 1994; Green et al. 1997a; Green et al. 1997c; Figure 6-1), glial cells (Bishop and Simpkins 1994), endothelial cells (Alvarez et al. 1997; Shi et al. 1997; Spyridopoulos et al. 1997), and osteoblasts (S. Manolagas, personal communication). Significant protection of RBCs from $FeCl_3$-induced lysis required a 20 nM estratriene concentration, and higher concentrations did not increase the magnitude of the cytoprotection (Figure 6-4b). The higher concentrations of estrogens required to protect RBCs suggest that nuclear events may contribute to the cytoprotective effects of the steroid in nucleated cells. The differences in effective estrogen doses may also be due to different mechanisms of cell death, as anuclear cells cannot undergo classic programmed cell death which, by definition, requires nuclear participation.

It is unlikely that the high concentrations of estrogens required for cytoprotection in this study are due to the absence of GSH in the experimental buffer, as RBCs contain high concentrations (about 2 to 2.5 mM) of GSH (Ćhernoch and Maliská 1966; Srivastava and Beutler 1969). The final concentration of GSH in the experimental solution should therefore be approximately 0.5 to 0.8 mM which is much higher than the 3.25 μM concentration with which a synergistic interaction was observed with estratrienes.

Summary

These results indicate that estrogens can attenuate oxidative-stress in neuronal cells in the absence of a nuclear ER. The potency of this estrogen-mediated neuroprotection is increased by the presence of sub-protective concentrations of GSH in

the media. Further, estrogens can protect RBCs from oxidative stress even though these cells are anuclear and cannot support a genomic mechanism of action. Together, these data support a non-genomic ER mechanism for the neuroprotective effects of estrogens.

Oxidative damage is a component of a variety of neurodegenerative diseases including AD, and age is associated with a decreased antioxidant capacity of the brain (Benzi and Moretti 1995). The possibility that either estrogens or antioxidants such as vitamin C or vitamin E can attenuate the disease process is presently being explored in several clinical trials. Our data suggest the possibility that phenolic A ring estrogens could be used for their neuroprotective properties without the estrogenic side effects such as feminization or an increased risk of certain cancers which are ER mediated. Further, these data suggest that a combination of estrogen therapy with antioxidant therapy may prove more effective than either alone.

CHAPTER 7
ESTRADIOL TREATMENT ENHANCES CREB PHOSPHORYLATION IN SK-N-SH NEUROBLASTOMA CELLS

Introduction

Estrogens exert many effects on neurons that cannot be explained by a classical genomic action of the steroid, including rapid effects on neuronal activity (Nabekura et al. 1986; Wong and Moss 1992), rapid induction of dendritic spine outgrowth (Briton 1993), rapid activation of MAPK activity (Singh et al. 1999), and transcriptional regulation of non-ERE containing genes (Szot and Dorsa 1994; Watters and Dorsa 1998). βE2 has been shown to activate the cAMP-PKA pathway in neuronal cells (Guanga et al. 1974; Weissman et al. 1975; Gu and Moss 1996), resulting in CREB phosphorylation (Gu et al. 1996; Zhou et al. 1996; Watters and Dorsa 1998). Further, activation of the cAMP-PKA pathway contributes to several effects of βE2, such as induction of dendritic spine outgrowth (Murphy and Segal 1997), increased expression of the neurotensin gene (Watters and Dorsa 1998), modulation of μ-opioid receptor (Lagrange et al 1997), and potentiation of kainate-induced currents (Gu and Moss 1996).

Activation of the cAMP-PKA pathway is associated with decreased susceptibility of neuronal cells to apoptotic signals (Rydel and Greene 1988; D'Mello et al. 1993, Kew et al. 1996; Campard et al. 1997). Walton et al. (1997) found that neurons which were vulnerable to ischemia exhibited an ischemia-induced decline in PO_4-CREB immunoreactivity whereas neurons which were resistant to the insult showed an ischemia-induced increase in PO_4-CREB immunoreactivity. Similarly, hypoglycemic seizure results

in a reduction of CREB immunoreactivity in susceptible neurons which precedes cell death in those areas (Panickar et al. 1997).

βE2 is a potent neuroprotective agent in both *in vitro* and *in vivo* models (for review see Simpkins et al. 1998). This study explores a possible role of CREB in the neuroprotective effects of estrogens. First, it was determined if αE2, which is as potent and efficacious as βE2 in neuroprotection assays (Behl et al. 1997b; Green et al. 1997a, Green et al. 1997c, Chapter 3, Chapter 6), can induce phosphorylation of CREB similar to the induction by βE2. Next, it was examined if serum deprivation alters CREB activity and if this effect is attenuated by βE2 treatment.

Materials and Methods

Cell Treatments

SK-N-SH cells were grown to approximately 75% confluency in Corning 100 mm dishes. Forskolin (Sigma Chemical Co., St. Louis, MO) was solubilized in DMSO and added to the cultures at 2.5 - 50 μM concentrations, and cells were exposed to DMSO (as a vehicle control) or forskolin for 30 min. Cells were exposed to either βE2 or αE2 at the indicated concentrations or 0.01% ethanol (as a vehicle control) for 1.5 h (Figures 7-5 and 7-7) or 3 h (Figure 7-6). βE2 and αE2 were initially dissolved 1 mg/ml in absolute ethanol and diluted to the final concentration in the culture media. For serum-deprivation experiments, experiment was initiated by rinsing the dishes with SF media twice then beginning the incubations either in SF media or SF media with 10 nM βE2. In all experiments, all dishes were photographed to verify confluency. Viability was assayed in the serum-deprivation experiments using the trypan blue dye exclusion technique as described in Chapter 2.

Protein Extraction

Nuclear and cytosolic protein was extracted at the end of treatment as described in Chapter 2. Some protein extractions were performed with the technical assistance of Eileen Monck and Jason Millard.

Immunoblot Blot Analysis

Following SDS-PAGE separation of 20 μg of protein in a Biorad Mini Protean gel electrophoresis apparatus, samples were transferred to a nitrocellulose membrane (0.2 μ pore size) at 100 V for 90 min using a Biorad MiniTrans Blot apparatus. Following a water rinse, the membrane was blocked with 3% non-fat dry milk in PBS at room temperature for 20 min (PO_4-CREB assay) or 40 min (CREB western and activating transcription factor 1 (ATF1) assays) and then incubated with the primary antibody in blocking buffer overnight at 4° C. Primary antibodies used were rabbit anti-PO_4-CREB (Upstate Biotechnology, Lake Placid, NY at a 1:3000 dilution), rabbit anti-CREB (Upstate Biotechnology at a 1:1000 dilution), and goat anti-ATF1 (Upstate Biotechnology at a 1:500 dilution). These antibodies are human, rat, and mouse compatible. The blot was then washed twice with water and exposed to secondary antibody (Amersham donkey anti-rabbit for CREB (at a 1:1000 dilution) and PO_4-CREB (at a 1:1000 dilution) blots or Dako anti-goat (at a 1:1000) for ATF-1 blots) for 90 min at room temperature. Following another washing step, the blot was exposed to Amersham ECL reagents according to manufactures directions and exposed to radiographic film.

A dye-conjugated molecular weight (MW) ladder (Biorad), and at least one sample from each treatment group was run on each gel. With this procedure, both 38 and 45

kilodalton (KD) bands were seen with the PO_4-CREB antibody, along with a single 45 KD band with the CREB antibody, and a single 38 KD band with the ATF-1 antibody. Data were expressed as relative densities of the bands, and a minimum of 5 replicates were tested in each group. Immunoblot analysis was completed with the technical assistance of Eileen Monck.

Electromobility Shift Assay

EMSA to determine specific CRE binding activity was performed on nuclear extracts as described in Chapter 2.

Immunocytochemisty

SK-N-SH cells were plated at a density of 2×10^4 cells/well in Nunc 8-well chamber slides. Cells were fixed in a 4% paraformaldehyde solution for 10 minutes at room temperature. Cells were then blocked with an 8% BSA/PBS solution for 1 h and were then exposed to a 1:500 dilution of rabbit anti-CREB antibody (Upstate Biotechnology) for 1 h. Following three 5 min PBS washes, cells were incubated with a FITC conjugated anti-rabbit IgG secondary (Amersham, Arlington Heights, IL) at a 1:1000 dilution for 1 h. Following three final PBS rinses, the slide was coverslipped using Anti-Fade reagent from Molecular Probes (Eugene, OR) and visualized using a Nikon diaphot fluorescent microscope.

Results

Assay Validation

CREB is a nuclear protein as shown by imunocytochemical staining (Figure 7-1a). CREB immunoreactivity appeared in immunoblot analysis of the nuclear protein extract

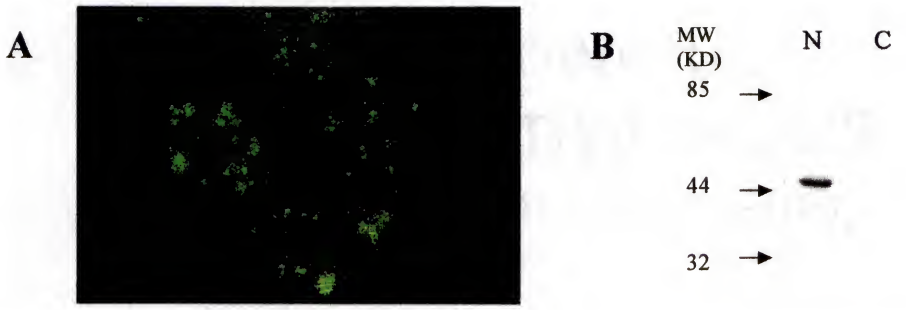


Figure 7-1. CREB is localized in the nucleus of SK-N-SH neuroblastoma cells. (A) depicts a photomicrograph of SK-N-SH cells (1000X magnification) which have been immunostained for CREB. (B) depicts an immunoblot of nuclear (N) and cytosolic (C) protein extractions from SK-N-SH cells.

but not in the cytosolic protein portion (Figure 7-1b). This verifies that the nuclear extraction procedure is effectively isolating nuclear protein.

Immunoblot analysis of SK-N-SH nuclear protein with the CREB antibody yields an apparent single band at a MW of 45 KD (Figure 7-1b, Figure 7-2) but which upon further resolution of the protein or lighter developing of the blot is revealed to be a duplet (data not shown). The CREB antibody recognizes both the α- and δ– isoforms of CREB

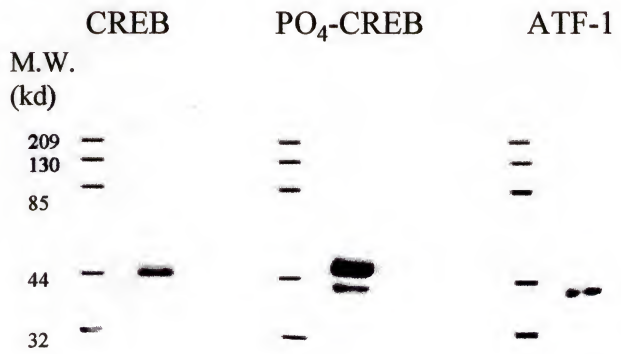


Figure 7-2. Comparison of CREB, PO_4-CREB, and ATF1 sizes and immunoreactivity in SK-N-SH cells. Protein from SK-N-SH cells was analyzed by immunoblot blot analysis with the CREB antibody, PO_4-CREB, or the ATF1 antibody from Upstate Biotechnology.

(Upstate Biotechnology, technical support service), and the duplet seen in the immunoblot blot are the proper MWs for these two CREB isoforms. Further verifying that this antibody detects both CREB isoforms, immunoblot analysis following two-dimensional electrophoresis confirmed that this antibody detected two proteins with the MW and pI values corresponding to the two isoforms of CREB (data not shown).

Immunoblot analysis of SK-N-SH nuclear protein with the PO_4-CREB antibody also shows two distinct bands at 45 KD and 38 KD (Figure 7-2). Upon further resolution, the 45 KD band is also a duplet (data not shown), and immunoblotting with the PO_4-CREB antibody following two-dimensional electrophoresis reveals that this immunoreactivity corresponds to two proteins with the MW and pI values which correspond to the α- and δ-isoforms of PO_4-CREB (data not shown). Throughout this chapter, PO_4-CREB immunoreactivity refers only to this 45 KD band.

PO_4-CREB is detected using a phospho-specific antibody to amino acids 124 to 136 of the human CREB sequence (Upstate Biotechnology, technical support service). This region is highly conserved among CREB family proteins (Lee and Masson et al. 1993), and Upstate Biotechnology reports that this antibody may also detect CREB family members ATF1 (with MW of 38 KD) and CREM (with a MW of 30 KD). The 38 KD band detected in the PO_4-CREB immunoblots most likely corresponds to ATF1, and ATF1 is detected in SK-N-SH cells at the expected MW (Figure 3-2). A 30 KD MW species is also seen on these immunoblots if the blots are exposed for longer intervals (data not shown).

Forskolin, a direct activator of AC, resulted in a dose-dependent increase in PO_4-CREB immunoreactivity but did not alter total CREB immunoreactivity (Figure 7-3). This dose-response relationship for forskolin-induced CREB phosphorylation is consistent with previously reported ED_{50} values (1-5 μM) for forskolin-activation of AC (Seamon et

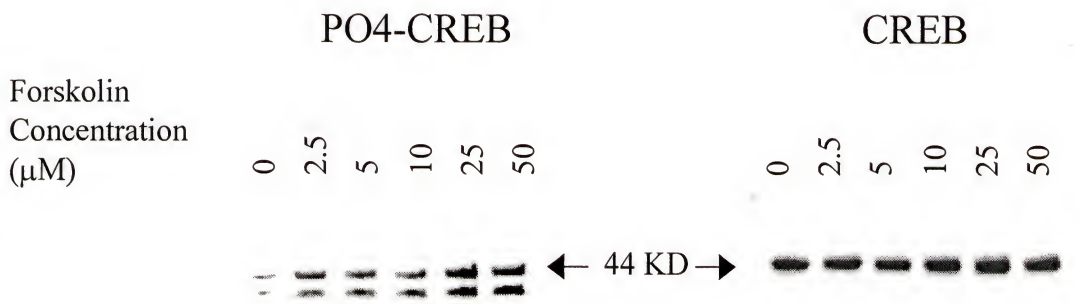


Figure 7-3. Forskolin causes a dose-dependent increase in PO_4-CREB immunoreactivity in SK-N-SH neuroblastoma cells but does not alter CREB immunoreactivtity. SK-N-SH cells were exposed to the indicated dose of forskolin for 30 min. DMSO (0.01%) was added to the 0 concentration groups. The immunoblots shown are representative of three repititions.

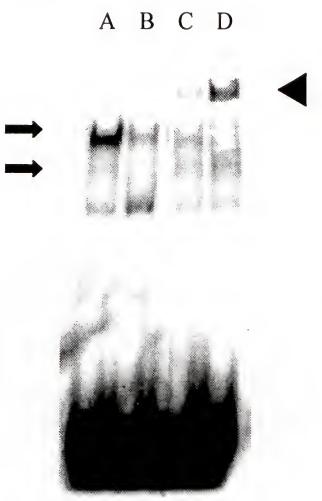


Figure 7-4. Optimization of electromobility supershift assay for CREB. 10 μg of SK-N-SH-N-SH nuclear protein was loaded into each reaction. Lane A contains the nuclear protein and labled CRE oligo. Excess unlabled CRE oligo was added to the reaction in Lane B. CREB antibody was added to the reactions in Lanes C (1 μl antibody) and D (2 μl antibody).

al. 1981) and forskolin-dependent phosphorylation of CREB (Montiminy and Bilezikjian 1987). This indicates that the immunoreactivity of the PO_4-CREB antibody is increased by treatments that increase CREB phosphorylation but the immunoreactivity of CREB antibody, which should detect CREB irrespective of phosphorylation status, is unchanged by increased phosphorylation of the protein.

EMSA revealed two specific CRE-binding bands and one non-specific band (Figure 7-4). Only the upper of the two specific bands supershifted with the addition of a CREB-specific antibody. Addition of 2 µl of CREB antibody resulted in a nearly complete shift in the upper band of CRE-binding activity.

Estradiol Treatment Increases CREB Phosphorylation in SK-N-SH Cells

A 90 min exposure to βE2 induced a dose-dependent increase in PO_4-CREB immunoreactivity. This effect was seen with as low as 1 nM βE2, and the maximal effect was seen with 10 nM concentration (Figure 7-5). The magnitude of this effect was a two-fold increase in basal CREB phosphorylation. This corresponds to an increase in phosphorylation of the CREB protein, as total CREB immunoreactivity was unchanged in all experiments (Figure 7-5). A similar increase is seen in the density of the presumed phospho-ATF1 band (Figure 7-5) with no increase in ATF1 immunoreactivity following 1 h of βE2 exposure (data not shown). This βE2-induced increase in CREB phosphorylation was not seen with less than 1 h exposure to the steroid (data not shown) but persisted through at least 3 h of βE2 exposure (Figure 7-6).

αE2 treatment increased CREB phosphorylation with a time course and dose-response similar to βE2 (Figure 7-6 and 7-7). A 1 nM dose of αE2 increased basal PO_4-CREB immunoreactivity by approximately 50 % in 90 min. The maximal response was a

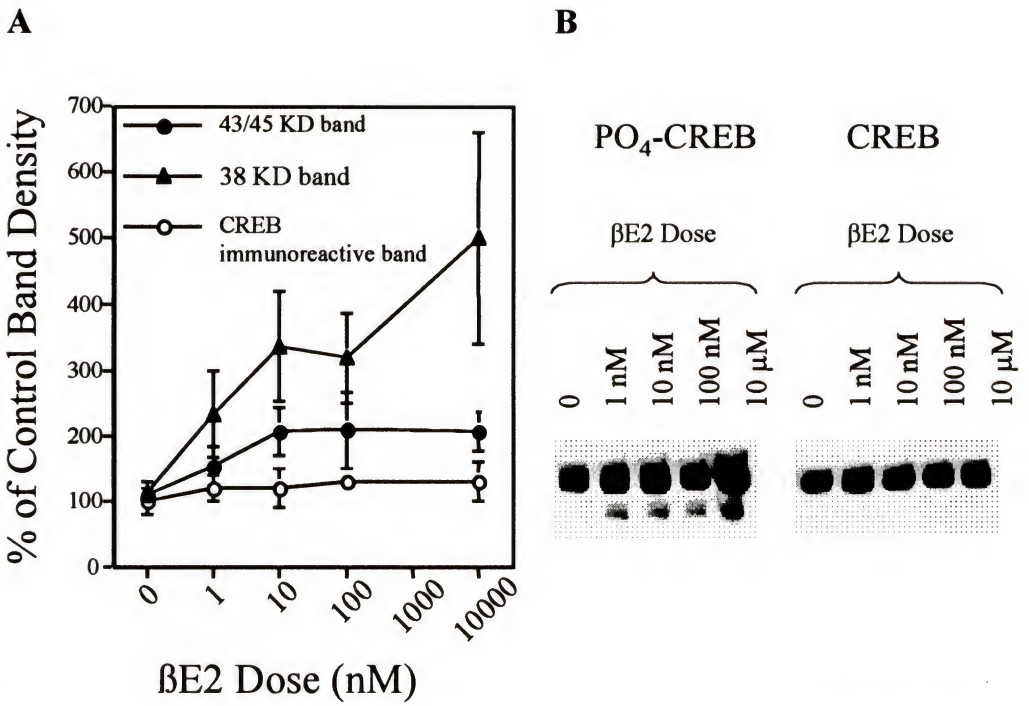


Figure 7-5 Effect of β-estradiol on CREB phsphorylation in SK-N-SH neuroblastoma cells. Cells were exposed to the indicated dose of βE2 or 0.01% ethanol (for vehicle control) for 90 min and CREB, and PO_4-CREB levels determined by immunoblot analysis. (A) depicts relative optical density for 5 to 6 dishes. The open circle corresponds to CREB immunoreactivity. The closed circle and closed triangle corresponds to immunoreactivity of the PO_4-CREB antibody corresponding to the MW of CREB (43/45 KD) and ATF1 (38 KD), respectively. A representative blot is shown (B).

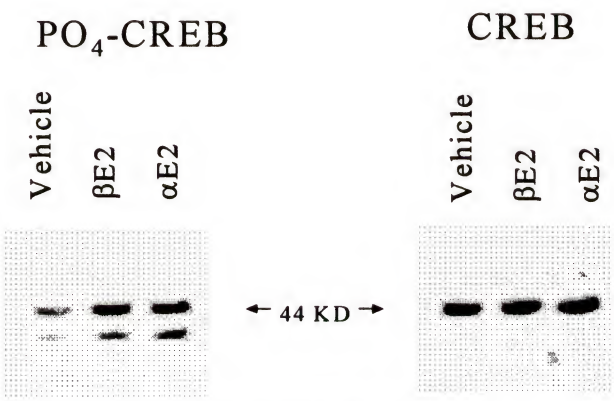


Figure 7-6. Effects of β-estradiol and α-estradiol on CREB phosphorylation in SK-N-SH neuroblastoma cells. Cells were exposed to 10 µM of either α- or β-estradiol or vehicle for 3 h. CREB and PO_4-CREB immunoreactivity was determined by Western analysis. Shown are blots representative of the 6 replications.

two-fold increase in PO_4-CREB immunoreactivity and this was seen between 10 and 100 nM αE2 concentrations. The presumed phospho-ATF1 band density was increased three- to four-fold with 100 nM αE2 exposure, and the dose response for this effect is identical to that for PO_4-CREB immunoreactivity (Figure 7-7). This αE2-induced CREB phosphorylation required at least 1 h of exposure and persisted out to 3 h exposure (Figure 7-6). At an 100 nM concentration, the magnitude of this effect was the same as the magnitude of the βE2-induced phosphorylation at either 1.5 h (Figure 7-5) or 3 h (Figure 7-6) based upon densitometric analysis. These short term treatments did not alter total CREB immunoreactivity (Figure 7-7).

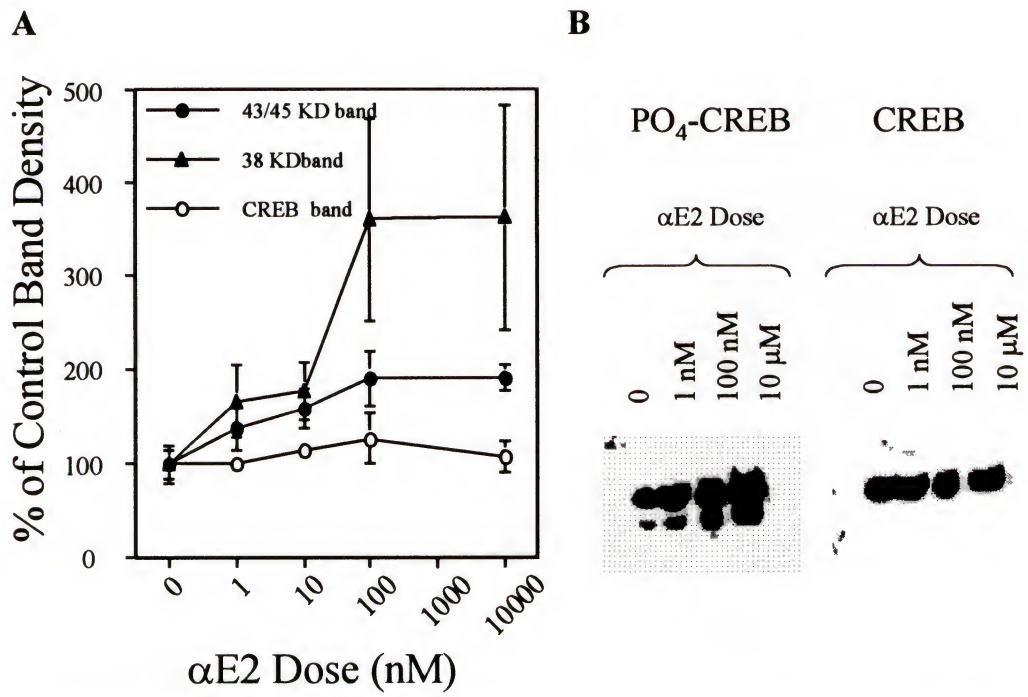


Figure 7-7. Effect of α-estradiol on CREB phsphorylation in SK-N-SH neuroblastoma cells. Cells were exposed to the indicated dose of αE2 or 0.01% ethanol (for vehicle control) for 90 min, and CREB and PO_4-CREB levels determined by immunoblot analysis. (A) depicts relative optical density for 5 to 6 dishes. The open circle corresponds to CREB immunoreactivity. The closed circle and closed triangle corresponds to immunoreactivity of the PO_4-CREB antibody corresponding to the MW of CREB (43/45 KD) and ATF1 (38 KD), respectively. A representative blot is shown (B).

Serum Deprivation and CREB

Serum deprivation resulted in a time-dependent decrease in CREB specific CRE binding activity which was seen by 4 h and persisted for at least 12 h (Figure 7-8). This reduction correlates with a decrease in total CREB band density which is reduced by an average of 47% by 4 h (Figure 7-9). The reduction in CRE binding activity was

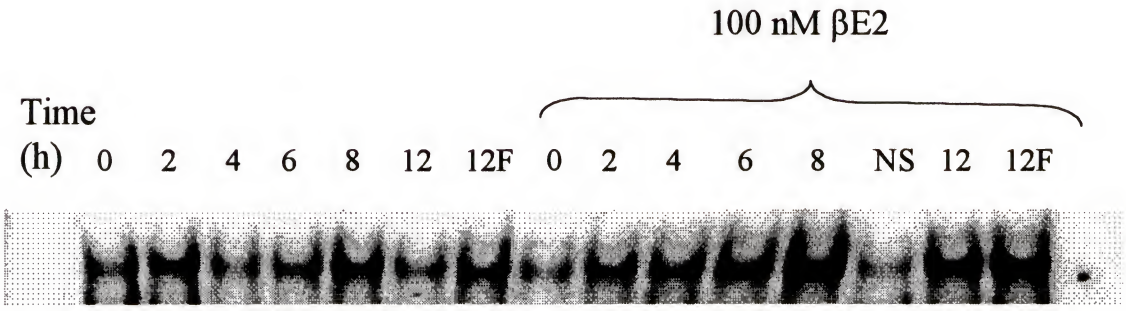


Figure 7-8. Effects of serum deprivation and β-estradiol treatment on CRE binding activity in SK-N-SH neuroblastoma cells. SK-N-SH neuroblastoma were serum deprived for the indicated length of time and the nuclear protein analyzed by EMSA analysis. 12F represents SK-N-SH cells which were retained in the presence of 10% FBS for the incubation period, and NS represents non-specific binding as determined by an excess of unlabled CRE oligonucleotides. Shown is a representative gel for the 4 repetitions of this experiment.

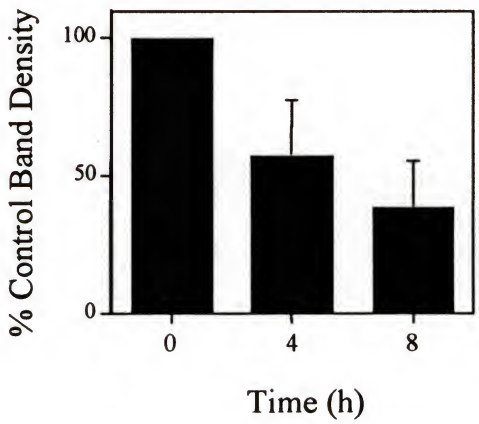


Figure 7-9. Effects of serum-deprivation on CREB immunoreactivity in SK-N-SH cells. Cells were serum-deprived for the indicated time period and total CREB was analyzed by immunoblot analysis. Depicted are mean relative optical density ± sem for 5 experiments.

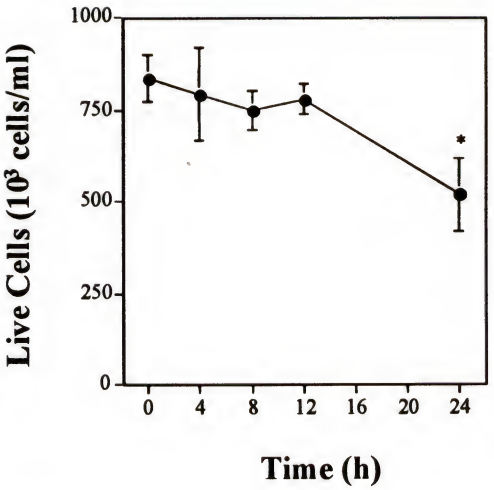


Figure 7-10. Time course for serum-deprivation toxicity on SK-N-SH neuroblastoma cells. Cells were plated at a densitiy of 1 x 10^6 cells/well and allowed to attach overnight. At time 0, all wells were rinsed twice with SF media and serum-deprived. At the indicated time, viability was determined by trypan blue dye exclusion. *<0.05 versus 0 time point. Depicted are mean ± sem for 3 wells per group.

attenuated by concomitant treatment with 10 nM βE2 (Figure 7-98. Interestingly, this reduction in CRE-binding activity and CREB immunoreactivity preceded the cell death induced by the toxicity (Figure 7-10).

Discussion

It was demonstrated that both the β- and α-isomers of estradiol can induce CREB phosphorylation in SK-N-SH neuroblastoma cells. Both isomers increase CREB phosphorylation at 1 nM concentrations and maximal stimulation of CREB phosphorylation is seen between 10 and 100 nM. Estradiol-induced phosphorylation of CREB is not seen with treatment times less that 1 h but persists for at least 3 h. The dose-response and time-course for both αE2- and βE2-mediated CREB phosphorylation is

consistent with that previously reported for βE2 in this cell line (Watters and Dorsa 1998) and in hypothalamic neurons (Gu et al. 1996).

The time-course and dose-response for this effect are also consistent with the time-course and dose-response for βE2-induced cAMP accumulation in SK-N-SH cells (Watters and Dorsa 1998) and hypothalamic neurons (Gunaga et al. 1974; Weissman et al. 1975) suggesting that the cAMP-PKA mediates the estrogen-induced CREB phosphorylation. CREB phosphorylation can also be mediated by the MAPK pathway (Xing et al. 1998) and CAMK pathway (Sheng et al. 1990). βE2 has been shown to increase both MAPK (Singh et al., 1999) and CAMK (Hayashi et al. 1994) activity in neurons in the same concentration range at which CREB phosphorylation is increased. An effect on βE2 on the activity of phosphotases cannot be excluded.

We also demonstrate both βE2- and αE2-induced phosphorylation of a 38 KD protein. This 38 KD protein is most likely ATF-1, as the antibody epitope is virtually identical between CREB and ATF-1 and the MW is consistent with ATF-1. ATF-1 has the exact DNA binding specificity as CREB (Hai et al. 1989; Hurst et al. 1991; Rehfuss et al. 1991) although the kinetics of the DNA binding vary (Hurst et al. 1991). Similar to CREB, ATF-1 is activated by phosphorylation of a serine residue (Flint and Jones 1991; Rehfuss et al. 1991). Following activation by PKA (Dash et al. 1991; Flint et al. 1991; Rehfuss et al. 1991) or CAMK (Sheng et al. 1990; Dash et al. 1991; Sheng et al. 1991), ATF-1 stimulates transcription of CRE-containing genes.

Serum deprivation reduces CREB protein level and CRE binding at time points preceding cell death as detected by trypan blue dye exclusion assays (Figure 7-10) and calcein AM/propidium iodide staining (data not shown). We have seen a similar decrease

in CREB levels in hippocampal slices within 1 h of anoxia (B. Jung, M. Hoffsteder, P.S. Green, J.W. Simpkins, unpublished observations). Further, a similar reduction in CREB levels preceding neuronal death has been reported following hypoglycemic seizures in female rats (Panikar et al. 1997) and ischemic injury in rats (Walton et al. 1997).

PO_4-CREB immunoreactivity decreases in a manner similar to CREB immunoreactivity following exposure to serum deprivation (P.S. Green and J.W. Simpkins, unpublished observations) or ischemic injury (Walton et al. 1997). This may be solely reflective of the decreased CREB levels or may indicate a simultaneous alteration in cAMP-PKA activity. Evaluation of cAMP levels or PKA activity in addition to CREB and PO_4-CREB immunoreactivity would clarify the effects of serum deprivation or ischemia on the overall pathway.

Elevation of cAMP levels is sufficient to promote survival of cerebellar granule neurons (D'Mello et al. 1993), primary rat sympathetic neurons (Rydel and Greene 1988), and spinal motor neurons (Hanson et al. 1998) in culture. Further, increased cAMP concentrations attenuate the neurotrophin withdrawal toxicity in septal cholinergic neurons (Kew et al. 1996), sialoglycopeptide toxicity in PC12 cells (Kobayashi and Shinozawa 1997), and opiate-induced apoptosis in murine neuroblastoma cells (Goswami et al. 1998). The initial reduction in CREB phosphorylation following an insult may mediate the vulnerability of those neuronal cells to that insult, since a decrease in phosphorylation of CREB is seen only in neurons which are vulnerable to the insult and precedes neuronal death (Walton et al. 1997; Panickar et al. 1997).

In summary, physiologically relevant concentrations of estradiol can increase CREB phosphorylation and preserve CREB activity during exposure to a toxic

environment. These observations point to additional interventional studies to determine: 1) which second messenger pathways are involved in estrogen-induced CREB phosphorylation, and 2) whether CREB and CREB phosphorylation is either necessary and/or sufficient for estrogen-mediated neuroprotection. Future studies utilizing pharmacological inhibitors of the PKA, MEK, and CAMK are necessary to elucidate the pathways by which estrogens enhance CREB phosphorylation. Further studies to clarify the role of this pathway in βE2-mediated protection include evaluation of βE2-mediated neuroprotection in the presence of PKA inhibitors or other inhibitors of CREB activity and evaluation of pharmacological activators of CREB activity such as forskolin for neuroprotective activity in these cells.

CHAPTER 8
GENERAL DISCUSSION

ERT is associated with numerous beneficial health effects including a reduction in risk for cardiovascular disease and decreased incidence of osteoporosis and associated bone fractures (Grady et al. 1992; Paganini-Hill 1995b). However, estrogen use is also associated with an increased risk of endometrial cancer which is virtually eliminated by progestin use (Grady et al. 1992; Paganini-Hill 1995b). Further, ERT may increase the risk of breast cancer although the evidence for this is conflicting (Dupont and Page 1991; Grady et al. 1992). ERT is associated with a significant reduction in all-cause mortality (Bush et al. 1987; Petitti et al. 1987; Hunt et al. 1990; Henderson et al. 1991; Stampfer et al. 1991; Lafferty and Fiske 1994). Grady et al. (1992) reports that long-term ERT with concurrent progestin use increases life expectancy for a 50 yr old white woman by 1 to 2 yrs depending upon other risk factors. Women at risk for breast cancer receive the least benefit in increased life expectancy from ERT (about 1 yr), and women at risk for coronary heart disease receive the most benefit (about 2.2 yrs). Similarly, Hillner et al. (1986) estimates that estrogen use results in a gain of 2.6 quality-adjusted yrs.

Role of Estrogens in Cognition, Memory, and Neurodegeneration

ERT is also associated with a number of clinically relevant neurological benefits. Several studies indicate that estrogen use increases performance on some tests of memory/cognition, in particular those which involve verbal memory (for review see Sherwin and Carlson 1997). Epidemiological evidence supports a role for ERT in

reducing the incidence of AD in post-menopausal women (for review see Henderson and Paganini-Hill 1997). Further, several small, clinical trials indicate that ERT improves cognitive functioning in AD patients (for review see Henderson and Paganini-Hill 1997). Estrogen may also decrease the extent of neuronal damage from stroke in humans (Schmidt et al. 1996) and in animal models (Shi et al. 1997; Simpkins et al. 1997; Dubal et al. 1998; Zhang et al. 1998).

There are several possible explanations for estrogen-effects on memory and cognition, including modulation of neurotransmitter function. Cholinergic impairment correlates with memory function (Perry et al. 1978) and occurs early in AD (Bartus et al. 1982). Estrogen has been shown to enhance cholinergic function in rats (Luine et al. 1975; Luine 1985; O'Malley et al. 1987; Gibbs 1994; Singh et al. 1994). Further, βE2 can reverse the learning impairment associated with scopalamine, a muscarinic antagonist (Dohanich et al. 1994). Monoamine oxidase activity is decreased with βE2 administration (Luine et al. 1975), and monoamine oxidase inhibitors are reported to modestly improve cognitive measures in demented patients (Sano et al. 1997).

Increased synaptogenesis is a fundamental morphological event in learning/memory functions (Jessel and Kandel 1993), and several studies link βE2 exposure to increased neurite outgrowth and synaptic formation (Toran-Allerand 1976, Toran-Allerand 1980; Wooley et al. 1990; Wooley and McEwen 1992; Briton 1993; Murphy and Segal 1996; Briton et al. 1997b). Neuritic formation appears to require NMDA receptor function (Wooley and McEwen 1994; Murphy and Segal 1996; Brinton et al. 1997a) and is not attenuated by ER antagonists (Briton et al. 1997a). Additionally, this neurotrophic effect of βE2 may be mediated by interactions with neuronal growth factors (Toran-Allerand

1992; Miranda et al. 1993; Singh et al. 1994, Singh et al. 1995; Sohrabji et al. 1995a, Sohrabji et al. 1995b) and their intracellular signaling mechanisms (Singh et al. 1999).

Neuroprotective Effects of Estrogens

Estrogens may reduce the risk and/or improve the outcome of neurodegenerative diseases by directly enhancing the survival of neurons. βE2 was first reported to exert direct neuroprotective effects on SK-N-SH human neuroblastoma cells under conditions of serum deprivation (Bishop and Simpkins 1994). Since then, neuroprotective effects of βE2 have been reported in ten different types of neuronal cells with fourteen different toxicities (see Table 8-1; Chapters 3-6; Behl et al. 1995; Goodman et al. 1996; Green et al. 1996; Skaper et al. 1996; Singer et al. 1996; Behl et al. 1997b; Blum-Degen et al. 1997; Briton et al. 1997b; Brooke et al. 1997; Green et al. 1997a, Green et al. 1997b; Green et al. 1997c; Mattson et al. 1997; Moo-Jung et al. 1997; Regan and Guo 1997; Weaver et al. 1997; Gridley et al. 1998; Sagara 1998; Singer et al. 1998; Zaulynov et al. 1999).

The effective concentrations for βE2-mediated neuroprotection range from a low of 0.2 nM (Bishop and Simpkins 1994; Green et al. 1997a) to a high of 50 μM (Weaver et al. 1997). The differences in effective βE2 concentrations may relate to the presence of GSH in the culture conditions (Green et al. 1997c; Gridley et al. 1998), differences between neuronal types and culturing conditions, differences in insult type/severity or the presence of ERs in the neurons. Additionally, while many studies do not require βE2-pretreatment to achieve neuroprotection (Bishop and Simpkins 1994; Goodman et al. 1996; Green et al. 1996; Blum-Degen et al. 1997; Green et al. 1997a, Green et al. 1997b;

Table 8-1. Summary of Reported Neuroprotection by β-Estradiol in Cultured Neurons.

Neuronal Type	Species	Toxicity	Reference
SK-N-SH	human	serum-deprivation	Bishop and Simpkins 1994 ;Green et al. 1997a; Green et al. 1997b
		Aβ	Green et al. 1996; Gridley et al. 1998
NT2	human	EAA	Singer et al. 1998
		H_2O_2	Singer et al. 1998
B103	human	Aβ	Moo-Jung et al. 1997
IMR32	human	FeSO4 and H_2O_2	Blum-Degen et al. 1997
PC12	rat	Aβ	Mattson et al. 1997
HT-22	murine	Aβ	Behl et al. 1995; Behl et al. 1997b; Green et al. 1997c
		BSO	Behl et al. 1997b
		H_2O_2	Behl et al. 1997b
		Haloperidol	Sagara 1998
Primary Hippocampal	rat	Aβ	Goodman et al. 1996
		EAA	Goodman et al. 1996; Weaver et al. 1997
		$FeSO_4$	Goodman et al. 1996
		Hypoglycemia	Goodman et al. 1996
		gp120	Brooke et al. 1997
		Mast cell activation	Skaper et al.,1996
Primary Neocortical	rat	Age in culture	Briton et al. 1997
		EAA	Singer et al. 1996; Zaulynov et al. 1999
		Anoxia	Zaulynov et al. 1999
		gp120	Brooke et al. 1997b
		Haloperidol	Sagara 1998
Cortical Primary	murine	EAA	Regan and Guo 1997
		Hypoxia	Regan and Guo 1997
		Hemoglobin	Regan and Guo 1997

Green et al. 1997c; Moo-Jung et al. 1997; Regan and Guo 1997; Weaver et al. 1997; Gridley et al. 1998), pretreatment with βE2 increases the neuroprotective potency of the steroid (Green and Simpkins, unpublished observations; C. Behl, personal communication). The studies presented in this dissertation required a dose-range of.2 to 200 nM of βE2 to achieve neuroprotection.

αE2 is more than 100-fold less potent than its optimal isomer, βE2, in classical measures of estrogenic action including uterotrophic stimulation (Korenman 1969) and MCF-7 cell proliferation (Wiese et al. 1997). Nevertheless, αE2 is as potent and efficacious as βE2 in neuroprotection assays (Chapters 3-6; Green et al 1996; Behl et al 1997b; Blum-Degen et al. 1997; Green et al 1997a; Green et al. 1997b; Green et al 1997c; Zaulynov et al. 1999). Similarly, αE2 exerts cytoprotective potency and efficacy equivalent to βE2 in RBCs (Chapter 6), cultured myoblasts (Green, Zaulynov and Simpkins, unpublished observations), and osteoblasts (S. Manologas, personal communication). While αE2 does not exhibit the classical genomic effects of βE2, αE2 has been shown to affect other cellular events including increasing intracellular calcium release in chicken granulosa cells (Morley et al. 1992), inducing rapid phosphorylation of ERK (M. Singh and C.D. Toran-Allerand, personal communication) and increasing phosphorylation of CREB (Green et al. 1998). Both the potency and efficacy of αE2 in these second messenger effects are equivalent to βE2. αE2 is also equivalent to βE2 in antioxidant properties (Blum-Degan 1997; Römer et al. 1998) and shows similar inhibition of oxidation-induced activation of NFκB (Chapter 5).

Several lines of evidence suggests that these neuroprotective effects of estradiol are not solely mediated by a classical ER-mediated mechanism. First, ER antagonists do

Table 8-2. Comparison of Relative Neuroprotecive Effect and Relative Potencies in ER Binding, Uterotrophic Growth Stimulation, and MCF-7 Cell Proliferation.

Steroid	% of βE2 Activity			
	Neuroprotection[1]	RBA	Uterotrophic[2]	MCF-7 Proliferation[3]
βE2	100	100	100	100
αE2	89	49[2]	1	<1
E-3-ol	102	79[3]	ND	1
Estrone	88	66[2]	29	2
Estriol	75	16[2]	1	3

% of βE2 neuroprotection refers to % protection of serum-deprived SK-N-SH cells with a 2 nM concentration. % RBA, uterotrophic growth stimulation, and MCF-7 cell proliferation are reported as relative potencies. Abbreviations used : RBA, relative binding affinity; ND, not determined. [1]Green et al. 1997b; [2] Korenman 1969; [3] Wiese et al. 1997.

not block the neuroprotective effect of βE2 (Green et al. 1997a; Regan and Guo 1997; Weaver et al. 1997). Estrogen-mediated neuroprotection is also not blocked by inhibitors of mRNA or protein synthesis (Goodman et al. 1996; Regan and Guo 1997). Furthermore, the SAR for estrogen-mediated neuroprotection (Green et al. 1997b; Behl et al. 1997b) differs markedly from the SAR for steroid binding to the ER (Anstead et al. 1997) resulting in many phenolic A ring estrogens, including αE2, which have low estrogenic potency but are potent neuroprotective steroids (Table 8-2). Finally, estrogens have been shown to attenuate oxidative stress-induced neuronal death in HT-22 cells, a neuronal cell line which lacks nuclear ERs (Behl et al. 1995; Green et al. 1997c).

Potential Mechanisms of Action for Estrogen-Mediated Neuroprotection

Classical Estrogen Receptor Activity

Although an ER is not required for the neuroprotective effects of estrogens, estrogen induction of ERE-containing genes may contribute to the neuroprotection if an ER is present. βE2 exposure increases expression of the neurotrophins BDNF (Singh 1995; Sohrabji et al. 1995b) and NGF (Singh et al. 1993) most likely by an ER-mediated mechanism (Sohrabji et al., 1995b; Toran-Allerand 1996). The anti-apoptotic bcl-2 gene contains an ERE in the promoter region, and the expression of bcl-2 protein is also increased with βE2 exposure (Garcia-Segura et al. 1998; Singer et al. 1998). Further, the βE2-mediated increase in bcl-2 expression in NT2 neurons is blocked by tamoxifen (Singer et al. 1998).

Activation of the MAPK Signal Transduction Pathway

The interaction between estrogens and neurotrophins is complex, with differential and reciprocal regulation of ERs and neurotrophin receptors (both p75 and trkA) by their ligands (for review see Toran-Allerand 1996). Interactions between ER activation and peptide growth factor signaling pathways have been described in non-neuronal cell types (Bunone et al 1996; Ignar-Trowbridge et al. 1996; El-Tanani and Green 1997; Lee et al. 1997; Karas et al. 1998). Similarly, estrogen activation of neurotrophin signal transduction pathway has also been described in SK-N-SH neuroblastoma cells (Watters et al. 1997) and neocortical explants (Singh et al. 1999). Activation of this pathway is rapid, occurring within 5 to 15 min after estradiol exposure (Watters et al. 1997; Singh et al. 1999). The ER antagonist, ICI 182,780, did not attenuate ERK phosphorylation

(Watters et al. 1997; Singh et al. 1999). The MAPK pathway is activated upstream of ERK phosphorylation, and most likely, activation of b-raf is cardinal (Singh et al. 1999).

Singh et al. (1999) hypothesize that a multimeric complex consisting of an ER and b-raf mediates the βE2-mediated activation of the MAPK pathway. In support of this, they have demonstrated that βE2 treatment results in a rapid increase in b-raf activity which co-immunoprecipitates with the ER. Interestingly, treatment with αE2 can also activate ERK phosphorylation in cortical explants in the same concentration range as βE2 (M. Singh and C.D. Toran-Allerand, personal communication). This is consistent with the effect being mediated by an ER-b-raf complex. Although αE2 is 100-fold less potent than βE2 at activating ER-ERE-dependent responses (Table 8-2; Korenman 1969; Wiese et al. 1997), αE2 has between 11 and 89% of the binding affinity as βE2 (Korenman 1969; Hahnel et al. 1973; Vanderkurr et al. 1993; Wiese et al. 1997).

Estrogen-induced activation of the MAPK pathway may contribute to, but is likely not the sole mechanism, of estrogen-mediated neuroprotection, particularly in SK-N-SH neuroblastoma cells. Although Watters et al. (1997) report stimulation of the MAPK pathway in SK-N-SH cells by βE2:BSA conjugates, a BSA vehicle control was omitted from their studies. Further, unconjugated βE2 was not evaluated (Watters et al. 1997). However, in a study of the effectiveness of βE2:BSA conjugates, Stevis et al. (1998) report that βE2:BSA as well as BSA alone stimulate ERK phosphorylation in SK-N-SH cells, but unconjugated βE2 does not.

Activation of cAMP-PKA-CREB Pathway

Estradiol activates the cAMP-PKA pathway in neurons (Guanga et al. 1974; Weissman et al. 1975; Gu and Moss 1996; Watters and Dorsa 1998) and increases phosphorylation of CREB (Gu et al. 1996; Zhou et al. 1996; Green et al. 1998; Watters and Dorsa 1998). This could contribute to the neuroprotective effects of estradiol, as activation of the cAMP pathway has been shown to be neuroprotective in a variety of neuronal cells (Rydel and Greene 1988; D'Mello et al. 1993; Kew et al. 1996; Campard et al. 1997), and increased CREB phosphorylation is associated with increased resistance to ischemic injury (Walton et al. 1997). βE2 increases CREB phosphorylation in a dose-range of 1 to 10 nM (Chapter 7), which overlaps the 0.2 to 2 nM dose-range in which βE2 attenuates serum-deprivation toxicity and Aβ toxicity in these cells (Bishop and Simpkins 1994; Green et al., 1996; Green et al. 1997a).

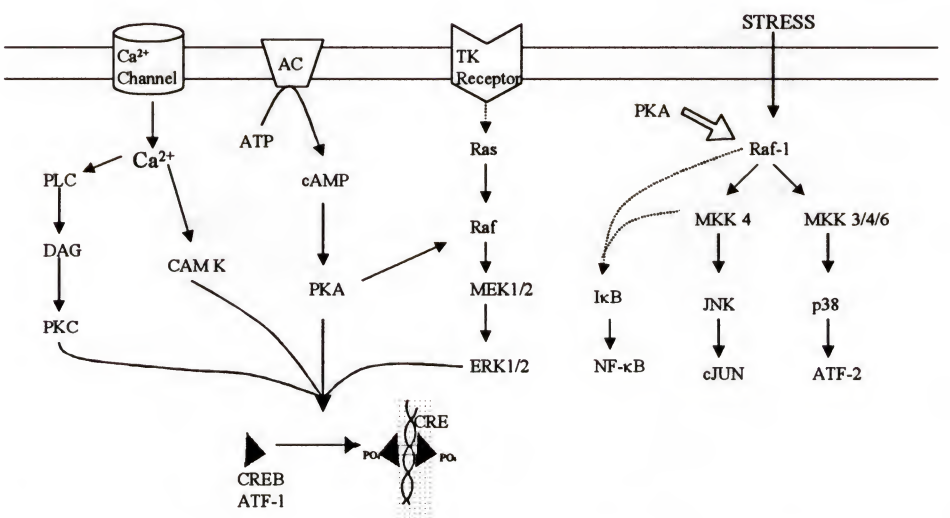


Figure 8-1. Schematic diagram of pathways involved in CREB phosphorylation. Open arrow represents inhibition of activity. All other arrows represent activation of the pathway.

The mechanism of the protective effects of the cAMP pathway in neurons is not known. PO_4-CREB may increase the expression of the anti-apoptotic protein bcl-2 (Ji et al. 1996). Additionally, there is significant cross-talk between the cAMP-PKA pathway and the MAPK pathways (Figure 8-1). PKA activity increases phorphorylation of b-raf which leads to ERK phosphorylation in PC12 cells (Vossler et al. 1997). Further, ERK activation has been shown to increase CREB phosphorylation in PC12 cells (Xing et al. 1998). PKA activity is also associated with inhibition of the pro-apoptotic raf-1 in non-neuronal cells (Zhang et al. 1997).

The cAMP-PKA-CREB pathway is known to mediate several effects of estradiol, including dendritic spine growth (Murphy and Segal 1997), increased expression of the neurotensin gene (Watters and Dorsa 1998), and potentiation of kainate-induced currents (Gu and Moss 1996). It remains to be elucidated if the cAMP pathway and, in particular, CREB is either necessary or sufficient to mediate the neuroprotective effects of estrogens.

Direct Attenuation of Glutamate Receptor Activation

Excitotoxic cell death occurs in several pathological disease states including stroke, AD, Huntington's disease, and AIDS-related dementia (for review see Lipton and Rosenberg 1994). This occurs when EAAs activate AMPA/kainate- and/or NMDA-type glutamate receptors and initiates sodium and calcium influx (for review see Choi 1992). Estrogens may exert neuroprotective effects by direct interaction with either AMPA/kainate or NMDA receptors. In electrophysiological studies, βE2 alters the depolarizing response to AMPA (Wong and Moss 1992). Weaver et al. (1997) report direct attenuation of NMDA-induced calcium currents with concurrent βE2 exposure. As

neuronal injury and energy depletion lead to toxic levels of extracellular glutamate (for review see Lipton and Rosenberg 1994), attenuation of excitotoxicity may confer protection from other toxicities. However, suppression of excitotoxicity cannot be the exclusive mechanism of estrogen-mediated neuroprotection. Neuronal cell types (SK-N-SH and HT-22 cells), which are not sensitive to AMPA/kainate or NMDA receptor-mediated toxicity (Zaulynov et al. 1999), are nevertheless protected from other toxicities by estrogens (Bishop and Simpkins 1994; Behl et al. 1995; Behl et al. 1997b; Green et al. 1997a; Green et al. 1997b; Green et al. 1997c; Sagara 1998).

Modulation of Intracellular Calcium Concentrations

Increased intracellular calcium concentrations modulate numerous neuronal functions including cell survival, synaptic formation and strength, and calcium-mediated neuronal death (for review see Ghosh and Greenberg 1995). Estrogens can modulate intracellular calcium levels through interactions with AMPA/kainate and/or NMDA receptors as discussed above (Wong and Moss 1992; Weaver et al. 1997). Additionally, βE2 treatment of ovariectomized female rats decreased L-type calcium currents in neostriatal neurons (Mermelstein et al. 1996), although voltage-gated calcium currents were increased in CA1 hippocampal neurons in a similar experimental paradigm (Joëls and Karst 1995). In cultured hippocampal neurons, βE2 exposure is associated with a modest increase in intracellular calcium (Murphy and Segal 1996). Further supporting βE2 modulation of intracellular calcium concentrations, βE2 treatment increases PKC activity in the preoptic area of female rats (Ansonoff and Etgen 1998) and biphasically modulates

CAMK activity in rat cerebelli *in vitro* (Hayashi et al. 1994), two calcium-triggered enzymes.

Antioxidant Activity

In both cell-free (Nakano et al. 1987; Sugioka et al. 1987; Mukai et al. 1990; Hall et al 1991; Mooradian 1993; Lacort et al. 1995) and cell culture (Goodman et al. 1996), phenolic A ring estrogens have been shown to be potent inhibitors of lipid peroxidation. The potency of these estrogens in these systems is equivalent to that of the well described antioxidant, α-tocopherol, tested under similar conditions (Nakano et al. 1997; Sugioka et al. 1987; Mukai et al. 1990; Hall et al. 1991). Several studies correlate the neuroprotective effects of estrogens with the steroidal antioxidant activity (Hall et al. 1991; Behl et al. 1995; Goodman et al. 1996; Behl et al. 1997b; Blum-Degan et al. 1998; Sagara 1998). βE2 can attenuate lipid peroxidation induced by Aβ exposure (Goodman et al. 1996; Gridley et al 1997), EAA toxicity (Goodman et al. 1996) or $FeSO_4$ exposure (Goodman et al. 1996; Blum-Degan et al. 1998). Further, estrogens attenuate the increase

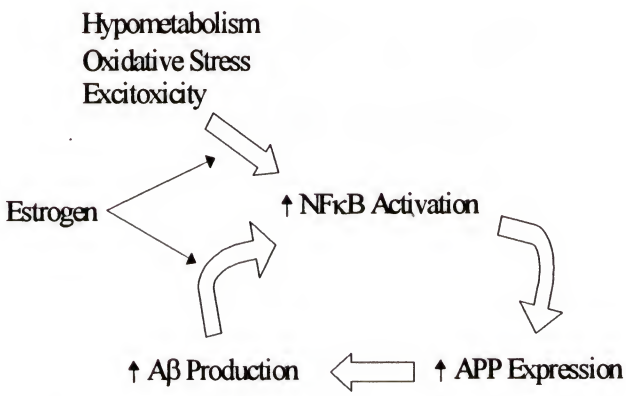


Figure 8-2. Potential interaction between NFκB activation and Aβ deposition and the role of estrogens in attenuating the process. Closed arrows indicate inhibition of the pathway.

in intracellular peroxides induced by Aβ (Behl et al. 1997b) and haloperidol (Sagara 1998).

Antioxidant activity of estrogens require concentrations of 1 to 10 μM in cell-free systems (Nakano et al. 1987; Sugioka et al. 1987; Mukai et al. 1990; Hall et al 1991; Mooradian 1993; Lacort et al. 1995). Similarly, antioxidant effects in cell culture models generally require more than 1 μM βE2 (Goodman et al. 1996; Behl et al. 1997b; Blum-Degan et al. 1998), and in these studies, the same concentration was required for the neuroprotective effects of βE2. Our laboratory has previously demonstrated diminished Aβ-induced lipid peroxidation with nM concentrations of βE2 which correlated with significant neuroprotection (Gridley et al. 1997). Interestingly, the neuroprotective potency of estrogens requires the presence of GSH in the media (Chapter 6; Green et al. 1997c; Gridley et al. 1998). The synergistic interaction could be due to redox cycling between endogenous antioxidants such as occurs between α-tocopherol and ascorbate (Sato et al. 1993), or ascorbate and GSH (Winkler et al. 1994).

Attenuation of Toxin-Induced NFκB Activation

NFκB activation is associated with several toxicities including glutamate exposure (Kaltschmidt et al. 1995; Grilli et al. 1996), Aβ exposure (Lezoualc'h and Behl 1997; Kaltschmidt et al. 1997), and hydrogen peroxide treatment (Schreck et al. 1991; Schmidt et al. 1995; Meyer et al. 1993; Lezoualc'h and Behl 1997). It is unclear if this activation of NFκB contributes to the toxicity of these treatments or occurs as a cellular protective mechanism. High levels of constitutive NFκB activity have been shown to confer resistance to oxidative stress (Lezoualc'h et al. 1998b). However, inhibition of oxidative

stress-induced NFκB activity by antioxidant treatment or by overexpression of the inhibitory protein IκBα imparts protection to the oxidative stress-induced toxicity (Grilli et al., 1996; Lezoualc'h et al.1998a; Post et al. 1998). Estradiol attenuation of H_2O_2-induced NFκB activation may contribute to the neuroprotective effects of the steroid; however, it may also be a marker of the antioxidant activity exerted by estradiol. The dose-range in which βE2 attenuates the H_2O_2-induced activation of NFκB (1 nM to 10 μM) in SK-N-SH cells overlap the dose-range in which estrogens attenuate H_2O_2-induced toxicity (1 to 100 nM) in the same cell line (Green and Simpkins, unpublished observations).

NFκB regulates the expression of many gene products, including APP (Grilli et al. 1995; Yan et al. 1995), α1-antichymotrypsin (Lieb et al. 1996), and IL6 (Ray et al. 1988) which are found in Alzheimer's plaques. Increased APP expression may lead to an increase in formation of Aβ peptide (Cechler 1995). Further, Aβ leads to increased activation of NFκB (Lezoualc'h and Behl 1997; Kaltschmidt et al. 1997). This cycle (Figure 8-1) could be initiated by an event such as an ischemic episode, which is known to increase NFκB activation (Clemens et al. 1997). Interestingly, cerebral infarct is a risk factor for AD (Kokmen et al. 1996).

Shi et al. (1998) have observed an increase in APP mRNA levels in the penumbral region of the lesion in rats following MCAO (Shi et al. 1998) and in hypoglycemic SK-N-SH cells (Shi and Simpkins, unpublished observations). In both instances, βE2 treatment alone did not alter APP mRNA levels in the absence of the toxicity but attenuated the toxin-induced increase in APP mRNA levels. Both ischemic insults (Clemens et al. 1997) and hypoglycemia (Tong and Perez-Polo 1995) are known to activate NFκB, and an

increase in APP expression could be a result of that activation. Similar to its effects on APP mRNA levels, estradiol treatment has no effect on NFκB activity in the absence of an insult but attenuates the toxin-induced activation of NFκB (Chapter 5). I hypothesize that estrogens may reduce Aβ accumulation by attenuating the initial NFκB activation following brain injury and NFκB activation due to Aβ deposition (Figure 8-2).

Summary

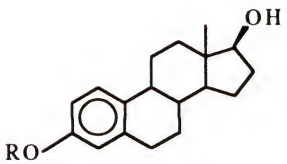
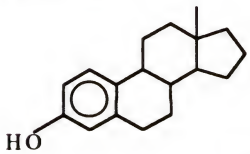
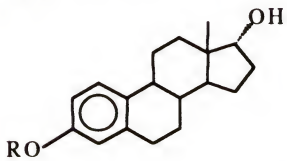
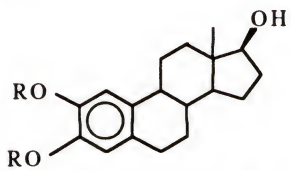
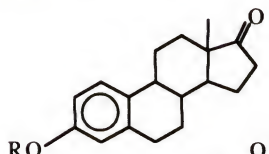
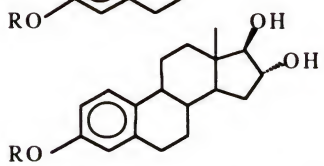
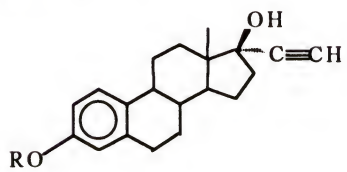
Estrogen is a multi-faceted hormone modulating many aspects of neuronal function. Several of these may contribute to the neuroprotective effects of estrogens. We and others have demonstrated that an ER is not necessary for the neuroprotective effects of estrogens. Evidence also suggests that while activation of the MAPK pathway may contribute to neuroprotection, it is not necessary for the neuroprotective effects of estrogens. It is unknown to what extent, if any, estrogen neuroprotection is dependent on the cAMP/PKA/CREB pathway, the NFκB pathway, antioxidant activity, or modulation of intracellular calcium concentrations. It is likely that some of these effects of estrogens are linked. For example, effects on membrane fluidity could modulate activity of membrane enzymes such as AC or ion channels such as NMDA receptors and other calcium channels. Similarly, the antioxidant activity of estrogens likely leads to modulation of NFκB activity. With the plethora of potentially neuroprotective pathways which are activated by estrogens, it is unlikely that a single pathway is solely responsible for the neuroprotective actions of the steroid. Further, the importance of each individual pathway could vary with neuronal type, developmental stage, type of receptors expressed,

extracellular environment, or other factors. Similarly, these factors may also alter the potency of estrogens in various models of neurotoxicity and neurodegeneration.

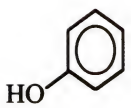
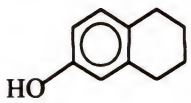
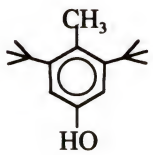
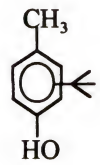
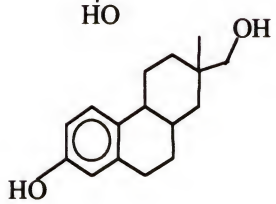
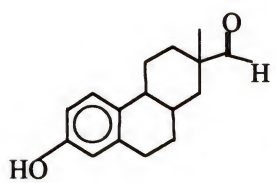
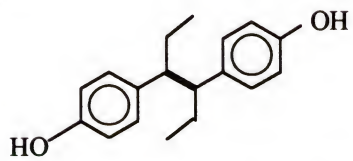
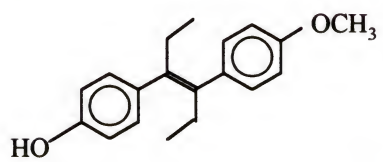
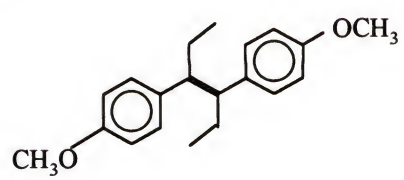
The neuroprotective effects of estrogens are dependent upon the presence of a phenolic A ring and can be disassociated from the classical estrogenic effects. These data suggests that a phenolic A ring estrogen without the feminizing effects could be effective in prevention of AD or in treatment of neurodegeneration and other forms of brain injury. Such a treatment would be useful for men as well as women for whom estrogen therapy is contraindicated.

APPENDIX
STRUCTURES OF COMPOUNDS EVALUATED FOR NEUROPROTECTION

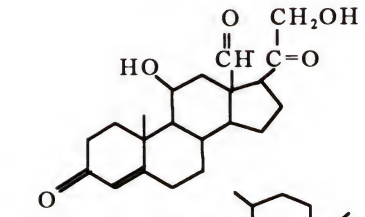
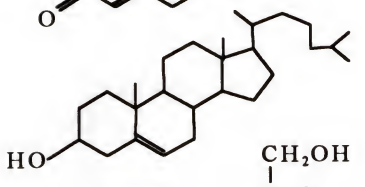
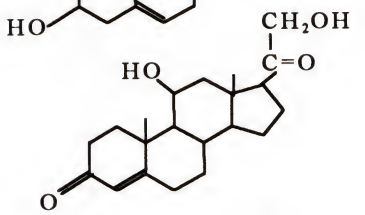
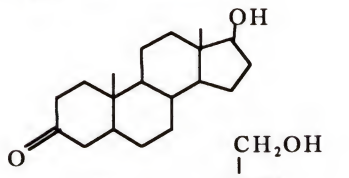
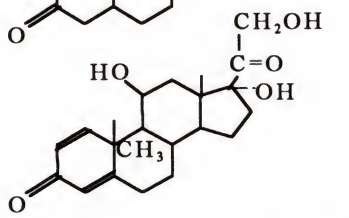
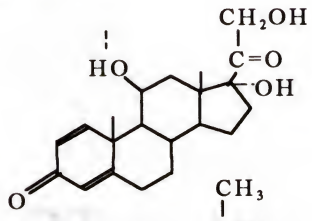
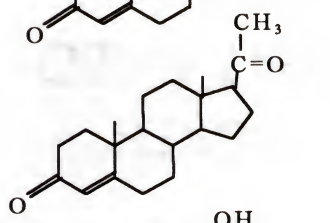
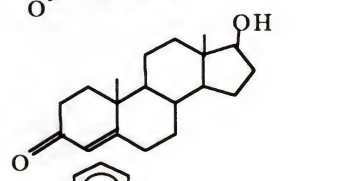
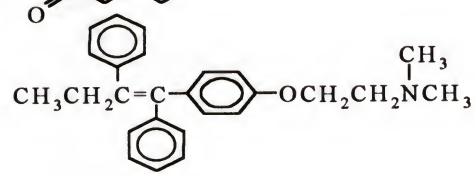
Estratrienes

Structure		Name
	R=H	17β-estradiol
	$R=CH_3$	17β-estradiol 3-O-methyl ether
		Estratriene-3-ol
	R=H	17α-estradiol
	$R=CH_3CO$	17α-estradiol 3-acetate
	R=H	2-hydroxyestradiol
	$R=CH_3$	17β-estradiol 2,3-O-methyl ether
	R=H	Estrone
	$R=CH_3$	Estrone 3-O-methyl ether
	R=H	Estriol
	$R=CH_3$	Estriol 3-O-methyl ether
	R=H	Ethynyl estradiol
	$R=CH_3$	Mestranol

Phenols

Structure	Name
	Phenol
	5,6,7,8-tetrahydronapthol
	Butylated hydroxytoluene
	Butylated hydroxyanisol
	Octahydro-7-hydroxy-2-methyl-2-phenanthrenemethanol
	Octahydro-7-hydroxy-2-methyl-2-phenanthrenecarboxaldehyde
	Diethylstilbesterol
	Diethylstilbesterol-mono-O-methyl ether
	Diethylstilbesterol-di-O-methyl ether

Other Steroids and Miscellaneous Compounds

Structure	Name
	Aldosterone
	Cholesterol
	Corticosterone
	Dihydrotestosterone
	Methylprednisolone
	Prednisolone
	Progesterone
	Testosterone
	Tamoxifen

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BIOGRAPHICAL SKETCH

Pattie S. Green was born in the old capital of the South, Richmond, Virginia, in 1971. After a childhood of much moving, her family settled down in Bradenton, Florida, where she graduated third in her class from Southeast High School, becoming the first member of her family to receive a high school diploma. Following a semester at the local community college (MCC) and an open heart surgery, she left Bradenton to attend the University of Florida.

For most of her college career, indecision ruled and Pattie could not pick a major. In 1994, as graduation approached, she was forced to a decision. She received her Bachelor of Science degree in microbiology and cell science, although she had also met the requirement for an English literature degree. She married her husband, Charles S. Flowers that year and spent that summer celebrating in Europe. Back from the honeymoon, Pattie found herself broke and in dire need of employment. Fortunately for her bank account, Dr. James W. Simpkins agreed to hire her in a technical position and she began to think seriously about graduate school. Fortunately for her, this decision was an easy one. In 1995, Pattie was accepted into the graduate program in pharmacodynamics and thus began a pleasurable academic journey under the guidance of Dr. James W. Simpkins. Several publications and a couple of research awards later, her career as a student is coming to an end. She is looking forward to a future filled with surprises, challenges, and hopefully, much happiness.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope, and quality, as a dissertation for the degree of Doctor of Philosophy.

Joanna Peris, Chair
Associate Professor of Pharmacodynamics

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope, and quality, as a dissertation for the degree of Doctor of Philosophy.

Ralph Dawson, Jr.
Associate Professor of Pharmacodynamics

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Maureen Keller-Wood
Associate Professor of Pharmacodynamics

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William J. Millard
Professor of Pharmacodynamics

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope, and quality, as a dissertation for the degree of Doctor of Philosophy.

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This dissertation was submitted to the Graduate Faculty of the College of Pharmacy and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

May 1999

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